(IFN)-γ axis named 'Mendelian susceptibility to mycobacterial disease (MSMD, MIM 209950)' (Dupuis *et al.*, 2000). Increased susceptibility to TB is also observed in this type of genetic disorders. Therefore, it is possible that mutations causing MSMD are responsible for the development of TB and/or that any functional polymorphisms of the genes encoding molecules of IL-12/IFN-γ axis may affect the genetic control of *M. tuberculosis*.

In the present study, we screened 21 candidate genes for TB susceptibility in Japanese by a gene-based association analysis using marker single nucleotide polymorphisms (SNPs) and subsequently analysed the association between TB and coding SNPs (cSNPs) adjacent to the positive marker SNPs in terms of susceptibility and disease severity.

Materials and methods

Subjects

The study population comprised 87 unrelated Japanese patients with TB (mean age: 52.7 ± 21.1 years; 18 women and 69 men) and 265 unrelated healthy Japanese individuals (mean age: 56.5 ± 12.7 years; 112 women and 153 men), who resided in Kyushu Island in the southern part of Japan. All the TB patients had been given a diagnosis of pulmonary TB on the basis of clinical symptoms and chest radiographic findings with bacteriological confirmation (culture, 82 patients; smear and/or polymerase chain reaction [PCR], 5 patients). Eleven patients were having TB relapses. Common clinical symptoms were cough (77%), sputum (53%) and fever (30%). Patients with known immunodeficient states, such as HIV infection and are undergoing immunosuppressive therapy were excluded. Lung disease on standard posterior–anterior chest radiograph of each patient was graded according the International Classification of Tuberculosis (Falk *et al.*, 1969; Van Lettow *et al.*, 2004):

(1) minimal lung disease was defined as infiltrates of slight to moderate density; disease present in a small portion of both lungs; the total volume of infiltrate(s) being the volume of one lung present above the second chondrosternal junction and the spine of the fourth junction or the body of the fifth thoracic vertebra and no cavitations present.
(2) moderately advanced disease was defined as disease present in one or both lungs; the total extending not more than as follows:
    (i) scattered lesions of slight to moderate density do not involve more than the total volume of one lung or the equivalent volume of both lungs
    (ii) dense, confluent lesions do not involve more than one-third of the volume of one lung, and
    (iii) the total diameter of the cavities are less than 4 cm; and
(3) far advanced lung disease was defined as: lesions more extensive than moderately advanced disease. Thirty-four, 38 and 15 patients had minimal, moderately advanced and far advanced lung disease, respectively. Twenty-nine patients had cavitary lesion(s). Subjects with diabetes were not included in the control group. After full explanation of the study by research personnel, written informed consent was obtained from the subjects or guardian(s). This study was approved by the ethical committees of Kyushu University and by the other participating institutions.

Screening of the candidate genes

Genomic DNAs were extracted from whole blood by using QIAamp DNA Blood Kit (Qiagen, Germantown, MD). Twenty-one candidate genes selected for analysis consisted of three genes whose association with TB has been observed in Japanese and/or other ethnic population (SLC11A1, VDR and IL-1β genes), 14 genes associated with IL-12/IFN-γ axis (IFN-γ, IFN-γR [IFN-γ receptor] P, IFN-γR2, IL-12 p40, IL-12p35, IL-12R [IL-12 receptor] β1, IL-12Rβ2, signal transducer and activator of transcription [STAT]-1, IL-18, IL-18R [IL-18 receptor], IL-23p19, IL-23R [IL-23 receptor], IL-27p28 and IL-27R [IL-27 receptor, WSX-1] genes), three genes associated with tumor necrosis factor (TNF)-α signaling (TNF-α, TNFRSF [TNF receptor superfamily]1 A and TNFRSF1B genes), and ubiquitin protein ligase E3A (UBE3A) gene, a putative TB susceptibility gene in chromosome 15q11–13 based on the sib-pair linkage analysis (Cervino *et al.*, 2002). All of them are located on autosomal chromosomes. HLA genes were not analysed in this study because of their complexity. These candidate genes were screened by association analysis of marker SNPs, which were validated by the TaqMan™ Validated SNP Genotyping Assays (Applied Biosystems, Foster City, CA). A total of 118 marker SNPs with 62–23 572 base pair (bp) interval within each gene (median 5633 bp interval) were genotyped by Assays-On-Demand™ primer and probe sets (Applied Biosystems) using ABI PRISM 7900HT (Applied Biosystems) according to the manufacturer's protocol.

SNPs detection and genotyping by PCR sequencing

For genes with two or more marker SNPs exhibiting significant allele association with TB (cut-off at $P < 0.05$), we subsequently searched for adjacent cSNPs by PCR and direct sequencing. Genomic DNAs extracted from whole blood of 24 TB patients randomly selected from the total TB population were used. Twenty-four samples are sufficient to detect SNPs with minor allele frequencies over 5%. To analyse exons 1–7 and 3′ UTR of *IL1B* adjacent to three marker SNPs with positive association (rs1143629, rs1143643 and rs3917368), we constructed eight pairs of oligonucleotide primer pairs according to the human *IL1B* gene sequence (GenBank Accession No. AY137079), as follows: 5′-AAACAGCGAGGGAGAAACTG-3′ and 5′-GCATACACACAAAGAGGCAGAG-3′ for exon 1, 5′-ACACATGAACGTAGCCGTCA-3′ and 5′-AGGGGAAAAATCTGGTCTCC-3′ for exon 2, 5′-GCAGGCTGTTTGCAGTTTCT-3′ and 5′-TCCTTGGGTTGGGAGTTAAA-3′ for exon 3, 5′-CTCCCTCCCTCGCTCTCT-3′ and 5′-CTGCCTGCTCTTGGCTAACT-3′ for exon 4,

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5′-CCTAAACAACATGTGCTCCA-3′ and 5′-AATTAG-CAAGCTGCCAGGAG-3′ for exon 5, 5′-CTGCACT-GCTGTGTCCCTAA-3′ and 5′-AAGTGGTAGCAGGA-GGCTGA-3′ for exon 6, 5′-CCTTGCCCCACAAAAATTC-3′ and 5′-TACCCTAAGGCAGGCAGTTG-3′ for 3′ UTR, and 5′-CTGGCAGAAAGGGAACAGAA-3′ and 5′-ACTTCTTGCCCCCTTTGAAT-3′ for 3′ UTR.

To analyse exons 1–17 of *IL12RB1* adjacent to two marker SNPs with positive association (rs2305739 and rs383483), we constructed 17 pairs of oligonucleotide primer pairs according to the human *IL12RB1* gene sequence (GenBank Accession No. AY771996), as follows: 5′-GCTTCAATGTGTTCCGGAGT-3′ and 5′-CCCACAGCTCTCCACACATA-3′ for exon 1, 5′-GAGGGTGCATAGATGGGAAA-3′ and 5′-ATCCT-CAGCCAACAATGAGG-3′ for exon 2, 5′-TGAGGTGA-CGCTGAAAGATG-3′ and 5′-TGAGGGTTTGGGAAT-GGTAG-3′ for exon 3, 5′-CACTGACACCCTCCTTC-CTG-3′ and 5′-CTGATGGCCTCTCTGGGTAA-3′ for exon 4, 5′-TTCAGGGCCCATTAACTCAC-3′ and 5′-CCTGGACTTGGGAAACAAAC-3′ for exon 5, 5′-TTCAGCACCAAAATGCAAAA-3′ and 5′-CTGAAC-TATGGGGCAGGGTA-3′ for exon 6, 5′-GGACAAT-TCTTACGGCCTGA-3′ and 5′-TTGCCCCTGTTCCTG-TACTC for exon 7, 5′-AGTTGGTTTGGTTCT-GATTGC-3′ and 5′-TCCCTCCATCTACCACTTGC-3′ for exon 8, 5′-TGCCTATGGGATGATGAGTG -3′ and 5′-GAGGCTCAGAGTAGGTGCTCA for exon 9, 5′-CAACTGTCTCGATGCGTCTC-3′ and 5′-AGGGC-ACAGAGGAGGGGTAG-3′ for exon 10, 5′-CCT-GGCCTTTGCTTATCCTT-3′ and 5′-CACTGTGCCC-AGCCTCTATT for exon 11, 5′-CCAGCATTCTTGGT-GTTGAC-3′ and 5′-CAGGTCTGCACTGCCTCAC-3′ for exon 12, 5′-CCTGGCCTCTGAGGAGTAAA-3′ and 5′-GCAGTGCATGCTGGGTAAAT-3′ for exon 13, 5′-AGGAAGAGGCAGGAGGTAGC-3′ and 5′-CTGC-CCAGCATCATTACCAT-3′ for exon 14, 5′-AGCAA-GACTCCGTCTCCAAA-3′ and 5′-AATGCGTAAC-CCTTGTCCAG-3′ for exon 15, 5′-GTGGCCCTA-CCCTCCCTCT-3′ and 5′-CTGACCGTCTGGCCCACT for exon 16, and 5′-CTACAACCACCCCCTGAAAG-3′ and 5′-CCATTTCATGGCAGCATCTA-3′ for exon 17.

Approximately 10 ng of genomic DNA and 5 pmol of each primer were used in a standard PCR reaction. Direct sequencing of PCR products was performed using the Big Dye terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's protocol. Sequencing reactions were run on an ABI 3700 automated sequencer (Applied Biosystems). Data were collected and analysed using the ABI DNA Sequencing Software Version 3.6. cSNPs were identified using the SeqMan II software version 4 (DNASTAR Inc., Madison, WI, USA). Among the cSNPs identified, non-synonymous cSNPs were selected for the second-round association study. Genotyping of 641A/G, 1094T/C, 1132C/G and 1573G/A SNPs of *IL12RB1* was performed by PCR and direct sequencing using primer pairs for exons 7, 10 and 13 listed in previous discussions. Positions given for the four cSNPs are those noted in relation to the transcription start site.

Statistics

Chi-square tests were employed to evaluate statistical differences in genotype distributions and allele frequencies of each SNP between TB and control groups. Genotype distributions of tested SNPs were compatible with the Hardy–Weinberg equilibrium. P values less than 0.05 were considered statistically significant. Linkage disequilibrium (LD) was evaluated by Lewontin's D′ (| D′ |) running all pairs of bi-allelic loci (Hedrick, 1987). All statistical analyses including haplotype estimation and association by χ^2 test were performed by using SNPAlyze version 3.2 software (DYNACOM, Mobara, Japan) (Tanaka *et al.*, 2003).

Results

A total of 118 marker SNPs listed in Table 1 were genotyped for 87 TB patients and 265 control subjects. Location of these marker SNPs in each gene was as follows: 57 SNPs in intron, 2 synonymous cSNPs, 1 non-synonymous cSNP, 3 SNPs in 5′untranslated region (5′UTR), 5 SNPs in 3′UTR, 23 SNPs in the upstream of the 5′ end of the first exon (5′ upstream) and 27 SNPs in the downstream of the 3′ end of the last exon (3′downstream). These marker SNPs covered the 21 candidate genes, and frequencies of the minor allele observed in control subjects were between 0.01 and 0.50 (average was 0.25). Association analysis revealed that seven SNPs showed a significant difference ($P < 0.05$) in the allele frequencies between the two groups; 3 in *IL1B* (rs1143629 [$P = 0.002$], rs1143643 [$P = 0.002$] and rs3917368 [$P = 0.049$]); 2 in *IL12RB1* (rs383483 [$P = 0.011$], rs2305739 [$P = 0.037$]); and 1 in *STAT1* (rs2280234 [$P = 0.004$]) and *TNFRSF1B* (rs496888 [$P = 0.007$]) (Table 1). With respect to *STAT1*, the distance between rs2280234 and its closest known cSNP, rs1803838 (chromosome position 191670871), is 4.8 kb, whereas rs2280235 with 1.4 kb distance from rs1803838 showed no association ($P = 0.680$). As to *TNFRSF1B*, rs496888 is located 14 kb upstream to exon 1, and marker SNPs closer to exon 1 (rs976881, rs616645 and rs474247) showed no association. Therefore, *STAT1* and *TNFRSF1B* with a single positive marker SNP were not further analysed.

Sequencing analysis of coding regions of *IL1B* and *IL12RB1* and 3′ UTR of *IL1B* adjacent to the marker SNPs with positive association showed one cSNP in exon 5 of *IL1B* and seven cSNPs in exons 4, 7, 10 and 13, and in 3′UTR of *IL12RB1*. Among them, four cSNPs of *IL12RB1* (641 A/G in exon 7, 1094T/C and 1132C/G in exon 10 and 1573G/A in exon 13) previously reported in Japanese population (Sakai *et al.*, 2001) were non-synonymous and were further analysed for association study (Fig. 1). As shown in Table 2, a significant difference in the genotype and allele frequencies between TB patients and controls was found for *IL12RB1*641 A/G, 1094T/C and 1132C/G SNPs ($P = 0.030$, $P = 0.013$ and $P = 0.013$, respectively). The genotype and allele frequencies of 1132C/G SNP were exactly the same as those of 1094T/C SNP. Genotype and allele frequencies of 1573G/A SNP

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Table 1. List of marker SNPs analysed in this study

Gene symbol	dbSNP ID[a]	Location	Chromosome position[b]	Minor allele frequency[c]	*P* value[d]
SLC11A1(NRAMP1)	rs4674301	5′ upstream	219,068,367	0.20	0.854
	rs2290708	intron	219,077,882	0.08	0.096
	rs1059823	3′ UTR	219,085,349	0.28	0.234
	rs2227255	3′ downstream[e]	219,093,286	0.36	0.527
VDR	rs11608702	3′ downstream	46,515,035	0.33	0.816
	rs1544410	intron	46,526,102	0.18	0.768
	rs2239183	intron	46,530,927	0.07	0.869
	rs2248098	intron	46,539,623	0.33	0.623
	rs2239180	intron	46,542,313	0.19	0.114
	rs1540339	intron	46,543,593	0.26	0.109
	rs2238138	intron	46,550,760	0.12	0.361
	rs1989969	intron	46,564,277	0.31	0.164
	rs3890733	intron	46,575,640	0.01	0.651
	rs10083198	intron	46,582,232	0.45	0.855
	rs4516035	5′ upstream	46,586,093	0.01	0.518
	rs7976091	5′ upstream	46,590,819	0.37	0.694
IL1B	rs3917368	3′ downstream	113,299,013	0.48	0.002*
	rs1143643	intron	113,304,533	0.48	0.002*
	rs1143629	intron	113,309,749	0.50	0.049*
	rs1143623	5′ upstream	113,312,060	0.38	0.052
	rs13032029	5′ upstream	113,316,646	0.45	0.055
IFNG	rs2193049	3′ downstream	66,833,189	0.49	0.510
	rs2069718	intron	66,836,429	0.10	0.594
IFNGR1	rs11914	Coding, synonymous	137,561,281	0.07	0.159
	rs2234711	5′ UTR	137,582,213	0.49	0.572
	rs1327474	5′ upstream	137,582,768	0.06	0.658
	rs608914	5′ upstream	137,588,731	0.39	0.394
IFNGR2	rs2284553	intron	33,698,565	0.28	0.601
	rs2266241	intron	33,702,920	0.48	0.722
	rs9808753	Coding, non-synonymous	33,709,182	0.47	0.784
	rs2834214	intron	33,715,576	0.18	0.640
	rs1532	intron[f]	33,726,836	0.03	0.114
	rs2284556	intron[g]	33,728,175	0.19	0.508
	rs11088252	3′ downstream[g]	33,737,563	0.17	0.586
	rs7282496	3′ downstream[g]	33,741,452	0.19	0.594
IL12A (p35)	rs2242382	intron	161,194,604	0.08	0.146
	rs668998	3′ downstream	161,198,253	0.28	0.836
IL12B (p40)	rs11135058	3′ downstream	158,667,095	0.24	0.086
	rs6870828	3′ downstream	158,671,090	0.24	0.320
	rs2288831	intron	158,682,591	0.46	0.489
IL12RB1	C_3057455_10	3′ downstream	18,021,464	0.17	0.320
	rs404733	3′ downstream	18,030,997	0.44	0.054
	rs383483	intron	18,032,886	0.41	0.011*
	rs2305739	intron	18,041,194	0.21	0.037*
	rs2305742	intron	18,052,441	0.20	0.118
	rs436857	5′ UTR	18,058,635	0.19	0.158
	rs2045387	5′ upstream	18,061,586	0.01	0.995
	rs7250425	5′ upstream	18,062,757	0.30	0.441
	rs273504	5′ upstream	18,076,247	0.31	0.462
IL12RB2	rs1546159	intron	67,500,447	0.22	0.875
	rs7518845	intron	67,523,001	0.24	0.557
	rs7535591	intron	67,529,168	0.23	0.709
	rs2252596	intron	67,545,522	0.22	0.680
	rs6685568	intron	67,567,318	0.23	0.671
STAT1	rs867637	3′ downstream[h]	191,651,888	0.27	0.243
	rs12987796	3′ downstream	191,656,373	0.23	0.148
	rs1914408	intron	191,665,482	0.31	0.561
	rs2280235	intron	191,669,336	0.41	0.680
	rs2280234	intron	191,675,605	0.18	0.004*
	rs2280232	intron	191,676,272	0.20	0.653
	rs2066805	intron	191,658,407	0.05	0.093
	rs2066802	Coding, synonymous	191,700,173	0.22	0.585
	rs1467199	5′ upstream	191,706,008	0.47	0.891

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Table 1. *Continued*

Gene symbol	dbSNP ID[a]	Location	Chromosome position[b]	Minor allele frequency[c]	*P* value[d]
IL18	rs3882891	intron	111,519,971	0.44	0.598
	rs1834481	intron	111,529,037	0.01	0.322
	rs4937113	intron	111,534,931	0.44	0.674
	rs2043055	5′ UTR	111,536,834	0.43	0.810
	rs360712	5′ upstream[f]	111,545,237	0.14	0.727
	rs795468	5′ upstream[g]	111,547,407	0.14	0.761
IL18R1	rs1861246	5′ upstream[k]	102,425,301	0.42	0.148
	rs12999364	5′ upstream	102,432,647	0.38	0.086
	rs11465567	5′ upstream	102,436,918	0.03	0.344
	rs1558627	intron	102,443,202	0.57	0.136
	rs1974675	intron	102,444,893	0.19	0.813
	rs2270297	intron	102,451,193	0.43	0.082
	rs3213733	intron	102,456,402	0.16	0.318
	rs2241116	intron	102,461,783	0.15	0.480
	rs2287033	intron	102,469,755	0.20	0.694
	rs3732127	3′ UTR	102,472,268	0.16	0.371
	rs1420094	3′ downstream	102,474,205	0.20	0.678
	rs3732124	3′ downstream	102,476,570	0.21	0.633
IL23A	rs2371494	5′ upstream	55,014,267	0.06	0.635
	rs2066808	3′ downstream[l]	55,024,240	0.06	1.00
	rs2066807	3′ downstream[m]	55,026,949	0.06	0.588
IL23R	rs1343151	intron	67,431,150	0.10	0.439
	rs10889677	3′ UTR	67,437,141	0.28	0.922
	rs4655531	3′ downstream	67,439,799	0.17	0.626
	C_2720245_10	3′ downstream	67,442,774	0.12	0.678
IL27(EBI3, p28)	rs40834	3′ downstream	28,417,894	0.28	0.767
	rs40835	3′ downstream	28,417,956	0.24	0.644
	rs181207	intron	28,421,031	0.13	0.183
IL27RA(WSX-1)	rs1982632	5′ upstream	14,000,004	0.19	0.051
	rs2306190	intron	14,023,676	0.39	0.169
	C_1878989_10	3′ downstream	14,033,779	0.12	0.179
	rs10415758	3′ downstream	14,033,921	0.35	0.462
TNF	rs1800683	5′ upstream[n]	31,648,050	0.36	0.482
	rs2857713	5′ upstream[o]	31,648,535	0.19	0.228
	rs1799724	5′ upstream	31,650,461	0.22	0.522
	rs361525	5′ upstream	31,651,080	0.03	0.430
	rs769178	3′ downstream	31,655,493	0.21	0.747
TNFRSF1A	rs740841	3′ downstream[p]	6,303,550	0.35	0.264
	rs2302350	3′ downstream[p]	6,306,014	0.29	0.132
	rs1860545	intron	6,317,038	0.18	0.369
	rs4149577	intron	6,317,783	0.46	0.159
	rs4149576	intron	6,319,376	0.19	0.295
TNFRSF1B	rs590368	5′ upstream	12,157,717	0.33	0.677
	rs496888	intron	12,167,072	0.16	0.007*
	rs976881	intron	12,168,020	0.15	0.294
	rs616645	intron	12,175,090	0.21	0.747
	rs474247	intron	12,180,441	0.37	0.573
	rs653667	intron	12,186,074	0.31	0.111
	rs5746053	intron	12,196,564	0.16	0.127
	rs1061631	3′ UTR	12,202,765	0.14	0.787
UBE3A	rs4906951	3′ downstream	23,126,764	0.02	0.740
	rs12443207	intron	23,141,250	0.36	0.937
	rs12907375	intron	23,151,415	0.36	0.769
	rs4906708	intron	23,169,072	0.36	0.701
	rs7496951	3′ UTR	23,222,396	0.36	0.735

NOTE: SNP, single nucleotide polymorphism; UTR, untranslated region. [a] When reference SNP (rs) number is not available, assays-on-demand® assay ID is shown. [b] chromosome position of SNP is from the DBSNP build 124 in the database of the National Center for Biotechnology Information (http://www.nabi.nlm.nih.gov/). [c] Minor allele frequencies observed in control samples are shown. [d] *P* values of χ^2 test in allele frequency differences are shown. Alternatively, [e] synonymous cSNP of *CTDSP1* gene, [f] SNP in 3′ UTR or [g] SNP in intron of *TMEM50B* gene, [h] SNP in intron of *GLS* gene, synonymous cSNP or [j] SNP in intron of *TEX12* gene, [k] SNP in intron of *IL1RL1* gene, [l] SNP in intron or [m] nonsynonymous cSNP of *STAT2* gene, [n] SNP in 5′ UTR or [o] nonsynonymous cSNP of *LTA* gene, [p] SNP in intron of *PLEKHG6* gene.

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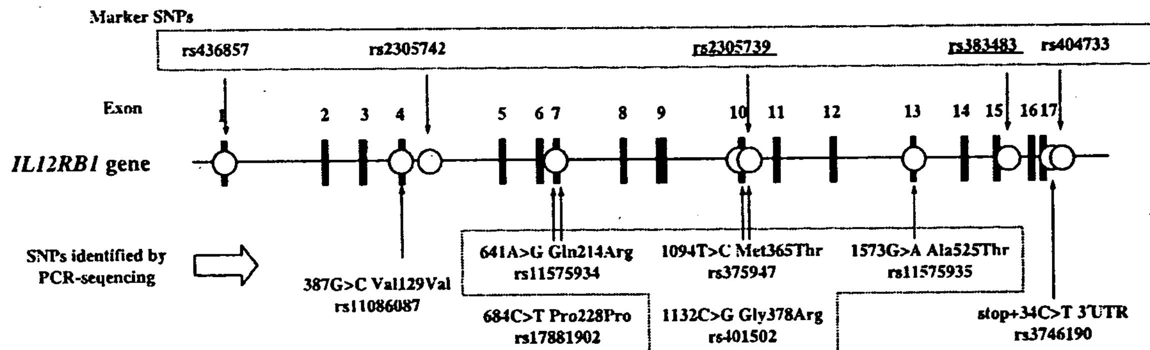

Figure 1. Structure of *IL12RB1* gene and location of the marker SNPs and identified cSNPs. SNP, single nucleotide polymorphism; UTR, untranslated region.

Table 2. Genotype and allele frequencies of *IL12RB1*641A/G, 1094T/C, 1132C/G and 1573G/A SNPs in TB patients and controls

IL12RB1 SNPs	Controls	TB	OR [95%CI]	*P*-value (chi-square)
641A/G				
Genotype frequency				
AA	98 (38%)	23 (27%)		
AG	120 (47%)	41 (48%)	1.46 [0.82–2.59]	0.20
GG	37 (15%)	22 (26%)	2.53 [1.26–5.08]	0.0078
Total	255	86		
Allele frequency				
A	316 (62%)	87 (51%)		
G	194 (38%)	85 (49%)	1.59 [1.12–2.25]	0.0087
1094T/C (1132C/G)				
Genotype frequency				
TT (GG)	96 (37%)	20 (23%)		
TC (GC)	125 (48%)	44 (51%)	1.69 [0.93–3.05]	0.080
CC (CC)	39 (15%)	23 (26%)	2.83 [1.40–5.73]	0.0032
Total	260	87		
Allele frequency				
T (G)	317 (61%)	84 (48%)		
C	203 (39%)	90 (52%)	1.67 [1.18–2.36]	0.0034
1573G/A				
Genotype frequency				
AA	1 (0%)	0 (0%)		
GA	15 (6%)	11 (13%)		
GG	247 (94%)	76 (87%)		
Total	263	87		
Allele frequency				
A	17 (3%)	11 (7%)	2.23 [0.93–4.40]	0.071
G	509 (97%)	153 (93%)		

NOTE: SNP, single nucleotide polymorphism; TB, tuberculosis.

were not significantly different between TB patients and controls (Table 2). When TB patients were divided into two subgroups according to the severity of lung disease, the advanced subgroup (patients with moderately or far advanced lung disease) showed prominent associations with GG genotype ($P = 0.0014$) and G allele ($P = 0.0015$) of 641 A/G SNP, and with CC genotype ($P = 0.00034$) and C allele ($P = 0.00044$) of 1094T/C or 1132C/G SNP (Table 3). There were no significant differences in the genotype and allele distributions of 641 A/G (genotype, $P = 0.48$; allele, $P = 0.36$) and of 1094T/C (1132C/G) (genotype, $P = 0.14$; allele, $P = 0.22$) between men and women of the control group (data not shown). Subsequent LD analysis of the four cSNPs spanning 12 kb of *IL12RB* showed almost complete LD among 641 A/G, 1094T/C and 1132C/G SNPs (D′ = 0.95–1.00) and modest LD between 1573G/A SNP and one of the other three SNPs (D′ = 0.64–0.81) (Table 4). To investigate if a particular haplotype constituted by these cSNPs was associated with the disease, haplotype frequencies were estimated and association analysis was performed. As shown in Table 5, the frequency of GCCC haplotype in TB patients

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Table 3. Genotype and allele frequencies of *IL12RB1* 641A/G, 1094T/C and 1132C/G SNPs in TB patient subgroups classified by disease severity

IL12RB1 SNPs	Controls	TB Minimal lung disease	OR [95%CI]	*P*-value (chi-square)	TB Advanced lung disease[a]	OR [95%CI]	*P*-value (chi-square)
641A/G							
Genotype frequency							
AA	98 (38%)	13 (38%)			10 (19%)		
AG	120 (47%)	14 (41%)	0.88 [0.39–1.96]	0.75	27 (52%)	2.21 [1.02–4.78]	0.041
GG	37 (15%)	7 (21%)	1.43 [0.53–3.85]	0.48	15 (29%)	3.97 [1.64–9.63]	0.0014
Total	255	34			52		
Allele frequency							
A	316 (62%)	40 (59%)			47 (45%)		
G	194 (38%)	28 (41%)	1.14 [0.68–1.91]	0.62	57 (55%)	1.97 [1.29–3.02]	0.0015
1094T/C (1132C/G)							
Genotype frequency							
TT (GG)	96 (37%)	12 (35%)			8 (15%)		
TC (GC)	125 (48%)	15 (44%)	0.96 [0.43–2.15]	0.92	29 (55%)	2.78 [1.22–6.36]	0.012
CC (CC)	39 (15%)	7 (21%)	1.44 [0.53–3.92]	0.48	16 (30%)	4.92 [1.95–12.4]	0.00034
Total	260	34			53		
Allele frequency							
T (G)	317 (61%)	39 (57%)			45 (42%)		
C	203 (39%)	29 (43%)	1.16 [0.70–1.94]	0.57	61 (58%)	2.12 [1.39–3.23]	0.00044

NOTE: Comparisons were made between controls and two subgroups of TB patients (minimal lung disease and advanced lung disease), respectively. SNP, single nucleotide polymorphism; TB, tuberculosis; [a], moderately or far advanced lung disease.

Table 4. Pairwise linkage disequlibrium analysis for four non-synonymous cSNPs of *IL12RB1* gene

	641A/G	1094T/C	1132C/G	1573G/A
641A/G		0.95	0.95	0.64
1094T/C			1.00	0.81
1132C/G				0.81

NOTE: SNP, single nucleotide polymorphism.
$n = 249$ (control samples), evaluated by absolute D′ static.

Table 5. Estimated frequencies of haplotypes constituted by four cSNPs of *IL12RB1* in TB patients and controls

	Frequency			
Haplotype[a]	Controls ($n = 249$)	TB ($n = 86$)	chi-square	*P*-value
ATGG	0.598	0.483	7.46	0.0063
GCCA	0.026	0.058	3.85	0.022
GCCG	0.339	0.436	5.23	0.050
others[b]	0.037	0.023		

NOTE: SNP, single nucleotide polymorphism; TB, tuberculosis.
[a] Haplotypes constituted by 641A/G, 1094T/C, 1132 G/C and 1573G/A.
[b] Haplotypes with frequencies < 0.03.

was higher than that in controls with a marginal significance ($P = 0.050$), whereas that of ATGG haplotype was significantly lower in TB patients than in controls ($P = 0.0063$).

Because the genotype information of 1094T/C and 1132C/G SNPs in *IL12RB1* was available in the database of International HapMap Project (http://www.hapmap.org), haplotype frequencies of the two loci in different ethnic groups were calculated and compared with those of our subjects. The frequency of CC haplotype of 1094T/C and 1132C/G SNPs in TB group (51.7%) was significantly higher than that in controls (39.0%) (odds ratio = 1.67, $P = 0.0034$), besides the frequencies in HCB (Han Chinese in Beijing, China, 38.7%), CEU (Utah residents with ancestry from Northern and Western Europe, 37.6%), supporting association of this haplotype of *IL12RB1* with TB.

Discussion

In a gene-based association study on 21 candidate genes for TB susceptibility using SNPs as genetic markers, we demonstrated that three non-synonymous cSNPs of *IL12RB1* were associated with TB in the Japanese population in terms of susceptibility and disease severity. Because direct association analysis using functional variants is limited by incomplete knowledge about functional variation at present, indirect association mapping using marker SNPs has been considered to identify genes conferring susceptibility to common diseases such as myocardial infarction and rheumatoid arthritis (Ozaki *et al.*, 2002; Tokuhiro *et al.*, 2003). We applied gene-based SNPs mapping to screen 21 candidate genes for TB susceptibility in the present study.

Two studies on Japanese population showed the association of *SLC11A1*and *IL12RB1* with TB, respectively. Gao *et al.* (2000) reported that a 5′ promoter $(GT)_n$ polymorphism of *SLC11A1* was associated with active TB in Japanese. On the other hand, Abe *et al.* (2003) found that a SNP in intron 4 (rs3731865) showing strong LD with 5′ promoter $(GT)_n$ did not affect TB susceptibility in Japanese.

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In the present study, although rs3731865 was not available from Assays-On-Demand™ primer and probe sets, an SNP (rs2290708) with a 2.4-kb distance from it showed no association with TB (Table 1). Akahoshi *et al.* (2003) reported that 641 A/G, 684C/T, 1094T/C and 1132C/G SNPs of *IL12RB1* in almost complete LD were associated with TB and one of the two common haplotypes (GTCC) was significantly associated with TB. The present study demonstrated a similar association as a result of gene-based screening of 21 candidate genes. We performed a haplotype analysis using different combination of SNPs including 1573G/A in exon 13 instead of 684C/T in exon 7. Although the difference in the frequencies of GCCG haplotype between TB patients and controls showed a marginal significance ($P = 0.050$), that of the protective haplotype, ATTG, was significant ($P = 0.0063$), assuring the association between *IL12RB1* and TB susceptibility. Therefore, our study, together with the study by Akahoshi *et al.* (2003), suggested that *IL12RB1* polymorphisms, at least in part, confer genetic susceptibility to TB in Japanese. When TB patients were divided into two subgroups according to the severity of lung disease, advanced subgroup of TB patients showed a prominent association with 641 A/G, 1094T/C and 1132C/G SNPs in the present study. Associations of HLA class II antigens and *SLC11A1* gene with severity of TB have been reported (Brahmajothi *et al.*, 1991; Rajalingam *et al.*, 1996; Kim *et al.*, 2005; Zhang *et al.*, 2005). This is the first report that suggests genetic variants of *IL12RB1* were associated with the progression to advanced forms of TB. In contrast, studies in Morocco and Korea did not demonstrate any association between the same cluster of SNPs and TB susceptibility (Remus *et al.*, 2004; Lee *et al.*, 2005). −2C/T SNP (rs436857), one of the two SNPs reported to be associated with pulmonary TB in the Moroccan study (Remus *et al.*, 2004), was included in the marker SNPs in the screening step of the present study, but no differences in the allele or genotype frequencies were observed between TB patients and controls ($P = 0.157$, Table 1). The difference between Moroccan and Korean studies, and Japanese ones including ours could be partly explained by the hypothesis that distinct environmental and natural selective factors resulted in population-specific immunogenetic adaptations to clinical TB (Stead, 1992; Delgado *et al.*, 2002). It is postulated that, in the area where TB has been endemic for a longer time, survivors were likely to be more resistant individuals. In the present study, the frequency of the resistant allele of 1094T/C (T allele) in controls was 0.61, which was lower than that in Moroccan study (0.74) (Remus *et al.*, 2004) and that in YRI (Yoruba in Ibadan, Nigeria) from the database of International HapMap Project (0.81).

To our knowledge, this is the first comprehensive association study of genes of IL-12/IFN-γ axis for TB susceptibility. IL-12/IFN-γ axis plays a pivotal role in the killing of intracellular mycobacteria. *IL-12RB1* encodes IL-12 Rβ1, one of the two subunits of receptor for IL-12, and is expressed on T and NK cells. Homozygous recessive mutations in *IL12RB1* preclude the surface expression of IL-12Rβ1 and IFN-γ secretion in vitro by otherwise functional T and NK cells (Altare *et al.*, 1998; de Jong *et al.*, 1998). The lack of IL-12-dependent IFN-γ secretion results in susceptibility to weakly virulent mycobacterial species, such as BCG and NTM despite the formation of mature granuloma through IL-12-independent IFN-γ secretion (Dorman & Holland, 2000; Casanova & Abel, 2002). One case of IL-12Rβ1 deficiency associated with the susceptibility to *Mycobacterium avium* complex was reported in Japan (Sakai *et al.*, 2001). The penetrance of IL-12Rβ1 deficiency for the MSMD phenotype is estimated to be less than 40% (Fieschi *et al.*, 2003), suggesting that the remaining patients could show different manifestation caused by related pathogens, such as TB. It is reported that patients with IL-12Rβ1 deficiency developed clinical TB in the absence of any personal or familial history of clinical disease by weakly virulent mycobacterial species (Altare *et al.*, 2001; Caragol *et al.*, 2003; Ozbek *et al.*, 2005). Akahoshi *et al.* (2003) demonstrated that CD2+ lymphocytes from healthy subjects homozygous for 641G, 1094C and 1132C haplotype corresponding to GCCG haplotype in the present study had a lower level of IL-12-induced signaling in vitro. Among the three cSNPs with positive association, 1132C/G (G378R) has been predicted to change the three-dimensional structure of the extracellular domain of IL-12Rβ1 through affecting the length of a predicted sheet (van de Vosse *et al.*, 2003). It is possible that this cluster of cSNPs is associated with functional change of IL-12R and directly affects the susceptibility to TB and progression of the disease in the Japanese population. As for polymorphisms of genes encoding IL–12, no association between rs3212227, an SNP in the 3′UTR of *IL12B*, and TB was demonstrated (Ma *et al.*, 2003). In the present study, both rs6870828 and rs2288831, which are located in the same LD block as rs3212227 based on the database of International HapMap Project, with 4.4 kb and 7.0 kb distance from it, respectively, were not associated with TB (Table 1).

In the screening step, two marker SNPs of *IL1B* in almost complete LD (rs3917368 in 3′ downstream and rs1143643 in intron) showed a significant association ($P = 0.002$), as shown in Table 1. With respect to rs3917368, advanced subgroup of TB patients showed a prominent association with the G allele ($P = 0.004$) and GG genotype ($P = 0.0084$) (data not shown). Although these SNPs are located outside the coding sequence, they might be associated with genetic susceptibility to TB or progression of the disease, through regulating the gene expression and/or alternative splicing, or being in strong LD with other functional SNP(s) in the non-coding region. Further study is needed to examine this possible association.

Limitations in this study include the correction for multiple comparisons and the power of the study to detect significant association, both resulting from a relatively small sample size. When Bonferroni correction was applied to the analysis in the screening step by multiplying cut-off value of 0.05 by 118, P values for the seven positive marker SNPs turned out to be not significant, necessitating confirmation by replication study. However, this study

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could serve as a replication of the previously observed association between the functional cSNPs and TB in the same ethnic population (Akahoshi *et al.*, 2003). Second, the statistical power to detect an OR of 1.6 at 0.05 significance level using 86 patients and 265 controls was 0.59 when the minor allele frequency in controls was 0.4 (Dupont & Plummer, 1990). Therefore, negative results on SNPs tested in this study do not necessarily exclude their association with TB.

In conclusion, gene-based association study on 21 candidate genes suggested that genetic variants of *IL12RB1*, at least in part, confer genetic susceptibility to TB, and are associated with the progression of the disease in Japanese. It would be warranted to examine whether the same association is observed in other ethnic groups.

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Selective predisposition to bacterial infections in IRAK-4–deficient children: IRAK-4–dependent TLRs are otherwise redundant in protective immunity

Cheng-Lung Ku,[1,2] Horst von Bernuth,[1,2,3] Capucine Picard,[1,2,19] Shen-Ying Zhang,[1,2] Huey-Hsuan Chang,[1,2] Kun Yang,[1,2] Maya Chrabieh,[1,2] Andrew C. Issekutz,[4] Coleen K. Cunningham,[5] John Gallin,[6] Steven M. Holland,[6] Chaim Roifman,[7] Stephan Ehl,[8] Joanne Smart,[9] Mimi Tang,[9] Franck J. Barrat,[10] Ofer Levy,[11,13] Douglas McDonald,[12,13] Noorbibi K. Day-Good,[14] Richard Miller,[15] Hidetoshi Takada,[16] Toshiro Hara,[16] Sami Al-Hajjar,[17] Abdulaziz Al-Ghonaium,[17] David Speert,[18] Damien Sanlaville,[20] Xiaoxia Li,[22] Frédéric Geissmann,[2,23] Eric Vivier,[24] László Maródi,[25] Ben-Zion Garty,[26] Helen Chapel,[27] Carlos Rodriguez-Gallego,[28] Xavier Bossuyt,[29] Laurent Abel,[1,2] Anne Puel,[1,2] and Jean-Laurent Casanova[1,2,21]

CORRESPONDENCE
Jean-Laurent Casanova:
casanova@necker.fr

Abbreviations used: B-EBV, EBV-transformed B lymphocyte cell line; CrP, C-reactive protein; IRAK-4, IL-1R–associated kinase 4; MDC, myeloid DC; MDDC, monocyte-derived DC; MIP-1β, macrophage inflammatory protein 1β; PDC, plasmacytoid DC; SV-40 fibroblast, SV40-transformed fibroblast; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRIF, TIR domain–containing adaptor-inducing IFN-β.

[1]Laboratory of Human Genetics of Infectious Diseases, U550, Institut National de la Santé et de la Recherche Médicale, 75015 Paris, France
[2]Necker Medical School, University Paris Rene Descartes, 75015 Paris, France
[3]Department of Pediatrics, Technical University Dresden, 01309 Dresden, Germany
[4]Department of Pediatrics, Dalhousie University, Halifax B3K 6R8, Nova Scotia, Canada
[5]Department of Pediatrics, Duke University Medical Center, Durham, NC 27710
[6]Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892
[7]Divison of Immunology and Allergy, Department of Pediatrics, Hospital for Sick Children, University of Toronto, Toronto M5G 1X8, Ontario, Canada
[8]Center for Pediatrics and Adolescent Medicine, University of Freiburg, 79106 Freiburg, Germany
[9]Royal Children's Hospital, Parkville, Victoria 3052, Australia
[10]Dynavax Technologies, Berkeley, CA 94710
[11]Division of Infectious Diseases and [12]Division of Immunology, Children's Hospital Boston, Boston, MA 02115
[13]Harvard Medical School, Boston, MA 02115
[14]Department of Pediatrics, Division of Allergy and Immunology, University of South Florida and All Children's Hospital, St. Petersburg, FL 33701
[15]3M Center, St. Paul, MN 55144
[16]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
[17]Department of Pediatrics, King Faisal Specialist Hospital and Research Center, Riyadh 11211, Kingdom of Saudi Arabia
[18]Centre for Infectious and Inflammatory Diseases, Child and Family Research Institute, Vancouver V5Z 4H4, British Columbia, Canada
[19]Immunodeficiency Investigation Center, [20]Laboratory of Histology, Embryology and Cytogenetics, Department of Molecular Cytogenetics and [21]Pediatric Hematology-Immunology Unit, Necker Hospital, 75015 Paris, France
[22]Department of Immunology, Cleveland Clinic Foundation, Cleveland, OH 44195
[23]U838, Institut National de la Santé et de la Recherche Médicale, 75015 Paris, France
[24]Centre d'Immunologie de Marseille-Luminy, Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Université Mediterranee, Campus de Luminy, 13288 Marseille, France
[25]Department of Infectious and Pediatric Immunology, Medical and Health Science Center, University of Debrecen, 4032 Debrecen, Hungary
[26]Department of Pediatrics, Schneider Children's Medical Center of Israel, Petah Tiqva 49202, Israel
[27]Department of Immunology, Nuffield Department of Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, England UK
[28]Department of Immunology, Hospital Universitario de Gran Canaria Dr. Negrin, 35020 Las Palmas de Gran Canaria, Spain
[29]Experimental Laboratory Medicine, University Hospital Leuven, 3000 Leuven, Belgium

C.-L. Ku and H. von Bernuth contributed equally to this work.
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Human interleukin (IL) 1 receptor–associated kinase 4 (IRAK-4) deficiency is a recently discovered primary immunodeficiency that impairs Toll/IL-1R immunity, except for the Toll-like receptor (TLR) 3- and TLR4-interferon (IFN)-α/β pathways. The clinical and immunological phenotype remains largely unknown. We diagnosed up to 28 patients with IRAK-4 deficiency, tested blood TLR responses for individual leukocyte subsets, and TLR responses for multiple cytokines. The patients' peripheral blood mononuclear cells (PBMCs) did not induce the 11 non-IFN cytokines tested upon activation with TLR agonists other than the nonspecific TLR3 agonist poly(I:C). The patients' individual cell subsets from both myeloid (granulocytes, monocytes, monocyte-derived dendritic cells [MDDCs], myeloid DCs [MDCs], and plasmacytoid DCs) and lymphoid (B, T, and NK cells) lineages did not respond to the TLR agonists that stimulated control cells, with the exception of residual responses to poly(I:C) and lipopolysaccharide in MDCs and MDDCs. Most patients (22 out of 28; 79%) suffered from invasive pneumococcal disease, which was often recurrent (13 out of 22; 59%). Other infections were rare, with the exception of severe staphylococcal disease (9 out of 28; 32%). Almost half of the patients died (12 out of 28; 43%). No death and no invasive infection occurred in patients older than 8 and 14 yr, respectively. The IRAK-4-dependent TLRs and IL-1Rs are therefore vital for childhood immunity to pyogenic bacteria, particularly *Streptococcus pneumoniae*. Conversely, IRAK-4-dependent human TLRs appear to play a redundant role in protective immunity to most infections, at most limited to childhood immunity to some pyogenic bacteria.

Inherited IL-1R–associated kinase 4 (IRAK-4) deficiency is an autosomal recessive disorder that was first described in three unrelated children (1). IRAK-4–deficient patients' fibroblasts and/or leukocytes show an impaired response to most Toll-like receptor (TLR) and IL-1R agonists tested (1–12). Specifically, the patients' whole blood cells or PBMCs do not respond to IL-1β, in terms of IL-6 secretion (1), or to IL-18, in terms of IFN-γ production (1, 4). Moreover, agonists of TLR1/2 (Pam_3CSK_4), TLR2/6 (Pam_2CSK_4), TLR3 (poly(I:C)), TLR4 (LPS), TLR5 (flagellin), and TLR9 (CpG DNA), do not induce the production of major inflammatory cytokines (TNF-α, IL-6, and IL-12) and growth factors (G-CSF and GM-CSF) in whole blood cells and PBMCs (1–9, 11, 12). However, the patients' PBMCs do respond to the nonspecific TLR3 agonist poly(I:C) and the TLR4-specific agonist LPS by producing IFN-β mRNA (for poly(I:C) and LPS) or IFN-α protein (for poly(I:C) only) (13). Moreover, the patients' fibroblasts have been shown to respond to poly(I:C) by inducing IFN-β, IFN-λ, and IL-6 (13). The human IRAK-4–independent TLR3/4 pathway is reminiscent of the mouse MyD88-independent, Toll/IL-1 receptor (TIR) domain–containing adaptor-inducing IFN-β (TRIF)–dependent TLR3/4 pathway (14, 15), which also controls the induction of cytokines other than IFNs, at least for TLR3 (16, 17). Despite the lack of IL-6 and TNF-α induction in response to poly(I:C) in human IRAK-4–deficient whole blood cells (1), the normal induction of IFN-α, -β, and -λ in response to poly(I:C) and LPS (13) raises the possibility that IRAK-4 deficiency may not prevent the induction of other cytokines in response to these two and possibly other TLR agonists.

The lack of response of IRAK-4–deficient whole blood cells and PBMCs to TLR and IL-1R agonists also does not exclude the possibility that individual leukocyte subsets may respond to at least some agonists. Several human leukocyte subsets produce TLR mRNAs and/or proteins. In the myeloid lineage, neutrophilic granulocytes express TLR1, 2, 4, 5, 6, 7, 8, and 10, as well as TLR9 upon induction with GM-CSF (18); monocytes express TLR1, 2, 4, 5, 6, 7, 8, and 9 (19–21); myeloid DCs (MDCs) express TLR1, 2, 3, 4, 5, 6, 7, 8, and 10 (22); and plasmacytoid DCs (PDCs) express TLR1, 6, 7, 9, and 10 (19, 21–23). Monocyte-derived DCs (MDDCs) express TLR1, 2, 3, 4, 5, 6, 8, 9, and 10, but hardly any TLR7 (24, 25). In basophilic and eosinophilic granulocytes, substantial expression has been confirmed only for TLR7 in eosinophils (26). In the lymphoid lineage, blood B cells express TLR1, 6, 7, 9, and 10 (20, 23, 27); NK cells express TLR1, 2, 3, 5, 6, 7, and 8 (20); CD4 α/β T cells express at least TLR1, 2, and 5 (28); and effector α/β CD8 T cells and γ/δ T cells express TLR3 (29, 30). In healthy controls, most subsets could be activated by the corresponding TLR agonists tested. In contrast, the range of blood cells in which TLR responses are affected by IRAK-4 deficiency remains unclear.

IRAK-4 deficiency may have an even broader impact, given the well-established role of IRAK-4 downstream from multiple IL-1Rs (1, 31) and the recently proposed role of IRAK-4 in TCR signaling (32). It is thus surprising that the first three patients identified were alive and well and had experienced only a few infectious diseases (1). To date, 21 IRAK-4–deficient patients have been reported in individual case reports or small series (1, 4–13, 33–36). Most presented with peripheral (e.g., pharyngotonsillitis, sinusitis, cellulitis, and endophthalmitis) and/or invasive bacterial diseases (e.g., meningitis, arthritis, septicemia, and visceral abscess) caused mostly by *Streptococcus pneumoniae* and *Staphylococcus aureus* (1, 4–13, 33–36). Only seven patients also presented infectious disease caused by Gram-negative bacteria (*Pseudomonas aeruginosa* in most cases) (1, 4–6, 8, 13, 33, 36). Although IRAK-4 deficiency appears to be more severe than initially thought (1), with seven reported deaths (5, 7–9, 13, 34, 36), the condition seems to improve with age, even without prophylaxis (4, 6, 36). The apparent broad resistance of IRAK-4–deficient patients challenges the prevailing view that TLRs are the principal sentinels of innate immunity (37–39). However, it has been difficult to draw firm conclusions in the absence of a large series of patients. Moreover, the rarity of infections may reflect the TLR-dependent, yet IRAK-4–independent, induction of certain cytokines in specific leukocyte subsets. We thus investigated the contribution of human TLRs to host defense by documenting

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the clinical course of a large number of IRAK-4–deficient patients and testing the TLR responses of their PBMCs for multiple cytokines, as well as the TLR responses of their individual leukocyte subsets.

RESULTS

IRAK4 mutations

We report 28 patients with IRAK-4 deficiency. The patients originate from 18 unrelated kindreds and 11 countries (Table I and Fig. 1). All *IRAK4* exons, flanking intron regions, and, when appropriate, entire introns, were sequenced in 24 patients (P1–4, 6–13, 15, 17–20, and 22–28). IRAK-4 deficiency was diagnosed on clinical grounds in four deceased relatives (P5, 14, 16, and 21) for whom no biological material was available. The patients of 13 kindreds were apparently homozygous (kindreds A–C, E, F, H–L, and P–R), and those from 5 kindreds were compound heterozygous (D, G, and M–O) for *IRAK4* mutations. However, four seemingly homozygous patients from three unrelated families (P2 from kindred B, P7 from kindred F, and P11 and 12 from kindred I) had one parent who did not carry the mutant allele. Fluorescence in situ hybridization with BAC210N13, which covers the entire *IRAK4* locus, and the genotyping of polymorphic markers showed that P2 was heterozygous for a large de novo deletion (designated BAC210N13del) encompassing *IRAK4* (Fig. S1, top, available at http://www.jem.org/cgi/content/full/jem.20070628/DC1; and not depicted). For P7, using the same BAC as for P2, fluorescence in situ hybridization revealed two signals, consistent with homozygosity owing to segmental uniparental disomy or compound heterozygosity with an undetected deletion encompassing a fraction of *IRAK4* (Fig. S1, bottom; and not depicted). Not enough material was available to explore the *IRAK4* locus in the deceased patients P11 and 12 from kindred I (8). 3 out of the 14 mutant alleles identified carried nonsense mutations (Y48X, Q293X, and E402X) (1, 3, 4, 6, 8, 9, 11, 36), 3 carried large deletions (*1-1096_40+23del*, *BAC210N13del*, and *942-1481_1125+547del*), 2 carried splice mutations (*1188+520A>G* and *1189-1G>T*) (12), and 6 carried frameshift insertions and deletions (*167_172insA*, *573delA*, *620_621delAC*, *631delG*, *821delT*, and *1240insA*) (1, 4, 7, 34) (Table I and Fig. 2 A). All mutations are predicted to be null, as they create a premature termination codon or delete a large segment of the gene. No missense mutation was found. The 14 mutations were not found in 100 healthy controls sequenced.

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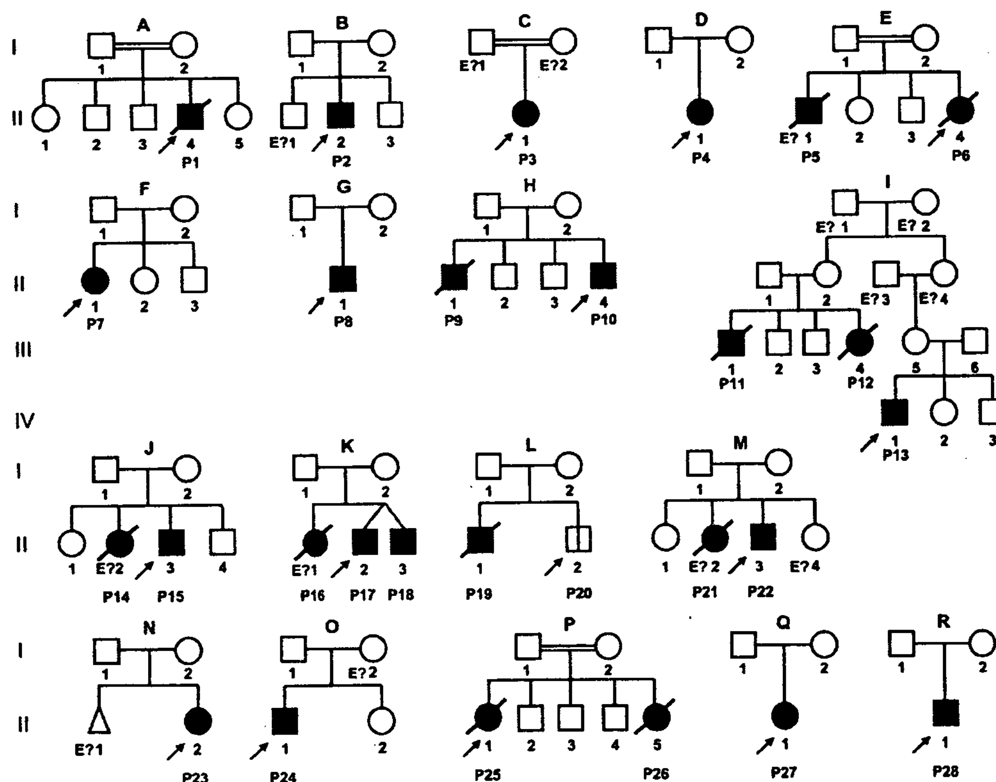

Figure 1. Pedigree of the 18 kindreds identified with IRAK-4 deficiency. Each kindred is designated by a capital letter (A–R), each generation is designated by a Roman numeral (I–IV), and each individual is designated by an Arabic numeral (from left to right). IRAK-4–deficient patients with a clinical phenotype are represented as closed symbols. P20, the only patient with confirmed IRAK-4 deficiency but no known clinical phenotype, is represented with an open square divided by a black line. In each family, the proband is indicated by an arrow. Individuals whose genetic status could not be evaluated are indicated by "E?"; they include four individuals (P5, 14, 16, and 21) thought to be IRAK-4 deficient based on their clinical phenotypes.

Table I. Genotypes, origin, and clinical phenotypes of IRAK-4–deficient patients

Kindred	Patient	Mutation	Origin	Follow-up	Age	Pathogens causing severe Gram-positive infections	Pathogens causing severe Gram-negative infections	References
A	P1 (II-4)	*821delT*	KSA	deceased	7 yr	Sp, Sa	-	(1, 13)
B	P2 (II-2)	Q293X/ *BAC210N13del*	Portugal	alive	14 yr	Sp, Sa	-	(1, 10, 13)
C	P3 (II-1)	Q293X	USA	alive	11 yr	Sp, Sa	Ec	(1, 3, 33)
D	P4 (II-1)	Q293X/ *620-621delAC*	USA	alive	24 yr	Sp, Cs	Nm	(2, 4, 35)
E	P5 (II-1)	ND	Turkey	deceased	16 mo	Sp, Spa	-	(34)
E	P6 (II-4)	*573delA*	Turkey	deceased	2 mo	Sp	-	(34)
F	P7 (II-2)	Q293X	UK	alive	32 yr	Sp	Ss	(6, 10, 13)
G	P8 (II-1)	*1188+520A>G/ 1189-1G>T*	Hungary	alive	9 yr	Sp	-	(10, 12, 13)
H	P9 (II-1)	Q293X	Canada	deceased	6 yr	Sp	Pa	(5, 9, 13)
H	P10 (II-4)	Q293X	Canada	alive	7 yr	Sp	-	(5, 9, 13)
I	P11 (III-1)	E402X	Spain	deceased	2 yr	Sa	Pa	(8, 13)
I	P12 (III-4)	E402X	Spain	deceased	8 mo	Sp	Pa	(8, 13)
I	P13 (IV-1)	E402X	Spain	alive	9 yr	Sp	-	(8, 10, 13)
J	P14 (II-2)	ND	Israel	deceased	3 mo	Sm	-	(13)
J	P15 (II-3)	*1-1096_40+23del*	Israel	alive	9 yr	Sp	-	(10, 13)
K	P16 (II-1)	ND	Canada	deceased	5 mo	Sa	-	(13, 36)
K	P17 (II-2)	Q293X	Canada	alive	27 yr	Sp	Pa	(13, 36)
K	P18 (II-3)	Q293X	Canada	alive	27 yr	Sp	-	(13, 36)
L	P19 (II-1)	*167_172insA*	Japan	deceased	2 yr	Sp	-	(7)
L	P20 (II-2)	*167_172insA*	Japan	alive	24 mo	-	-	(7)
M	P21 (II-2)	ND	USA	deceased	4 mo	bacterial meningitis	-	this study
M	P22 (II-3)	Q293X/ *620-621delAC*	USA	alive	10 yr	Sp, Sa	-	this study
N	P23 (II-1)	Y48X/ *631delG*	Canada	alive	2 yr	Sa	-	this study
O	P24 (II-1)	*1240insA/ 942-1481_1125+547del*	Canada	alive	16 yr	Sp, Sa	-	this study
P	P25 (II-1)	Q293X	Australia	deceased	4 mo	Sp	-	this study
P	P25 (II-5)	Q293X	Australia	deceased	6 mo	Sp, Sa	-	this study
Q	P27 (II-2)	Q293X	USA	alive	11 yr	Sp	-	this study
R	P28 (II-1)	Q293X	USA	alive	6 yr	Sp	-	(11)

Cs, *Clostridium septicum*; Ec, *E. coli*; KSA, Kingdom of Saudi Arabia; Nm, *N. meningitidis*; Pa, *P. aeruginosa*; Sa, *S. aureus*; Sm, *S. milleri*; Sp, *S. pneumoniae*; Spa, *S. parasanguis*; Ss, *S. sonnei*.

The Q293X mutant allele was found in homozygotes from six kindreds (C, H, K, P, Q, and R) and compound heterozygotes from four kindreds (B, D, M, and possibly F). The recurrence of this mutation may reflect a mutational hotspot, a founder effect, or both (unpublished data).

IRAK-4 expression and function

We assessed *IRAK4* mRNA levels in EBV-transformed B lymphocyte cell lines (B-EBVs; Fig. 2 B) derived from most patients and a healthy control by RT-PCR. The two patients carrying the *573delA* mutation died before cell lines could be established (34). Most other patients lacked detectable full-length *IRAK4* mRNAs species, presumably because of nonsense-mediated mRNA degradation. However, P7 (mutation Q293X), P8 (mutations *1188+520A>G* and *1189-1G>T*), P13 (mutation E402X), P19 (mutation *167_172insA*), and P22 (mutation Q293X/*620-621del*) had low levels of detectable full-length *IRAK4* mRNA. We then assessed IRAK-4 protein levels in B-EBVs (Fig. 2 C). No IRAK-4 protein was detected in any of the patients tested, even in P7, 8, 13, 19, and 22, all of whom had detectable full-length mRNAs, excluding a potential role of IRAK-4 as a scaffold protein in our patients (40, 41). Finally, we assessed the functional impact of *IRAK4* mutations. B-EBVs bearing mutations *821delT* (P1), Q293X (P2, 3, and 7), *1188+520A>G/1189-1G>T* (P8), E402X (P13), and *1-1096_40+23del* (P15) did not respond to TLR7 and 8 agonists, as measured by TNF-α production (Fig. 3 A). SV40-transformed fibroblasts (SV40-fibroblasts)

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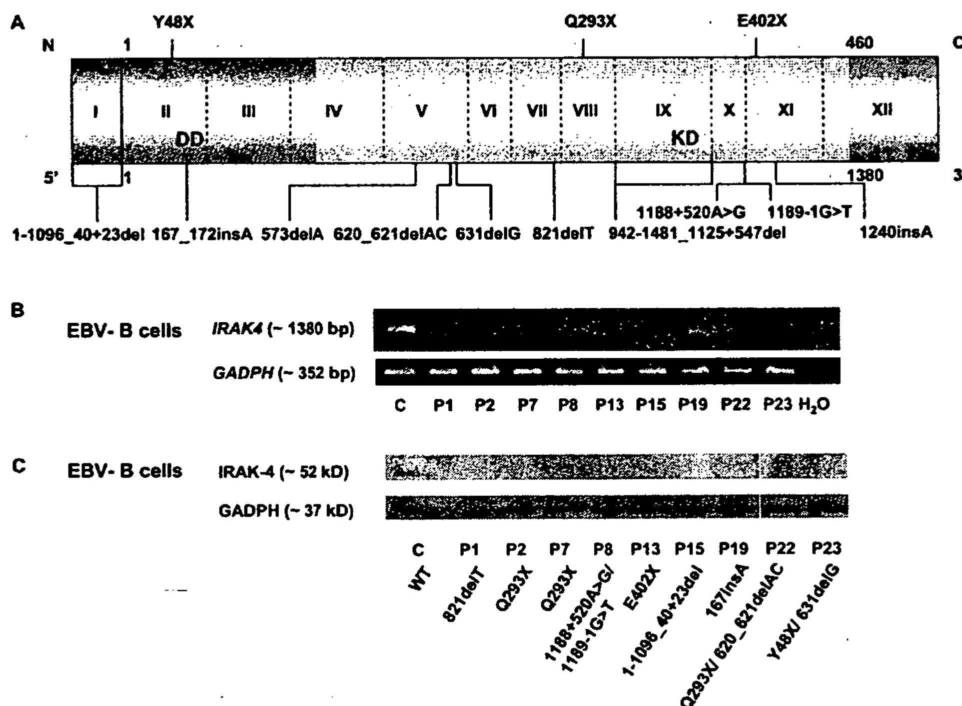

Figure 2. IRAK-4 deficiency. (A) Schematic representation of *IRAK4* with all identified mutations. The gene is composed of 12 exons, with exon 1 and a part of exon 12 noncoding. The N-terminal death domain (DD) and C-terminal kinase domain (KD) are shown in light gray. (B) RT-PCR of the full-length *IRAK4* and *GAPDH* genes in B-EBVs from a healthy control (C) and nine IRAK-4-deficient patients. (C) IRAK-4 and GAPDH protein levels in B-EBVs from a healthy control and nine IRAK-4-deficient patients, as shown by Western blotting. White lines indicate that intervening lanes have been spliced out.

bearing mutations *821delT* (P1), Q293X (P2 and 3), *1188+520A>G/1189-1G>T* (P8), E402X (P13), *1-1096_40+23del* (P15), Y48X/*631delG* (P23), and *1240insA/942-1481_1125+547del* (P24) did not respond to IL-1β, as assessed by measuring IL-6 production. However, IRAK-4–deficient SV40-fibroblasts did produce IL-6 upon activation by poly(I:C) (Fig. 3 B) (13). Thus, all patients had complete IRAK-4 deficiency and a complete absence of IRAK-4–dependent TIR signaling, owing to the inheritance of two loss-of-expression, loss-of-function *IRAK4* alleles.

Development and function of blood leukocyte subsets

We analyzed blood leukocyte subsets in 12 IRAK-4–deficient patients. We previously showed that granulocytes, $CD14^+$, $CD16^+$, and $CD14^+/CD16^+$ monocyte subsets, and MDCs and PDCs, were present in normal numbers in three patients (13). We now report that T cell subsets, including $CD4^+$ and $CD8^+$, and $CD45RA^+$ and $CD45RO^+$ T cells, are also present in normal numbers (Table S1, available at http://www.jem.org/cgi/content/full/jem.20070628/DC1), with the possible exception of normal to low levels of T cells in P17 and 18 (36). T cells proliferated normally in response to the mitogen PHA, CD3, and recall antigens in vitro (Table S2). B cells and memory B cells ($CD27^+$) were also present in normal numbers (Table S1). Serum Ig levels for IgA were normal in five, high in two (P8 and 11), and low in four (P1, 2, 17, and 18) patients (36). IgG levels were normal in seven and high in four (P7, 8, 11, and 17) patients, and IgM levels were normal in seven, high in three (P7, 11, and 19), and low in one (P2) patients. IgE levels were high in 8 (P1, 7, 8, 11, 13, 15, 17, and 23) out of the 11 patients evaluated (Table S2). Antibody responses to protein antigens were normal in all but two patients, who had slightly low titers (P7 and 15); however, the date of recall vaccination before serological testing was unknown. The antibody response to glycans was impaired in some (P2, 8, 17, 18, and 29) but not all patients, and in response to some but not all pneumococcal and erythrocyte AB antigens (Table S2 and unpublished data) (11, 12, 33). Finally, the surface expression of CD16 and CD56 on NK cells was normal (Table S1). IFN-γ secretion and surface expression of CD107 (degranulation) by the patients' NK cells were normal (unpublished data). Overall, there seemed to be no overt defect of leukocyte development in IRAK-4–deficient patients. Thus, antigen-specific T and B cell responses seemed to be normal, except for an impaired glycan-specific antibody response in at least some patients and against some glycans, and except for an overproduction of IgE in most of the patients tested.

Impaired production of multiple cytokines by blood mononuclear leukocytes

We previously reported that IRAK-4–deficient whole blood cells and PBMCs produce only very small amounts of TNF-α,

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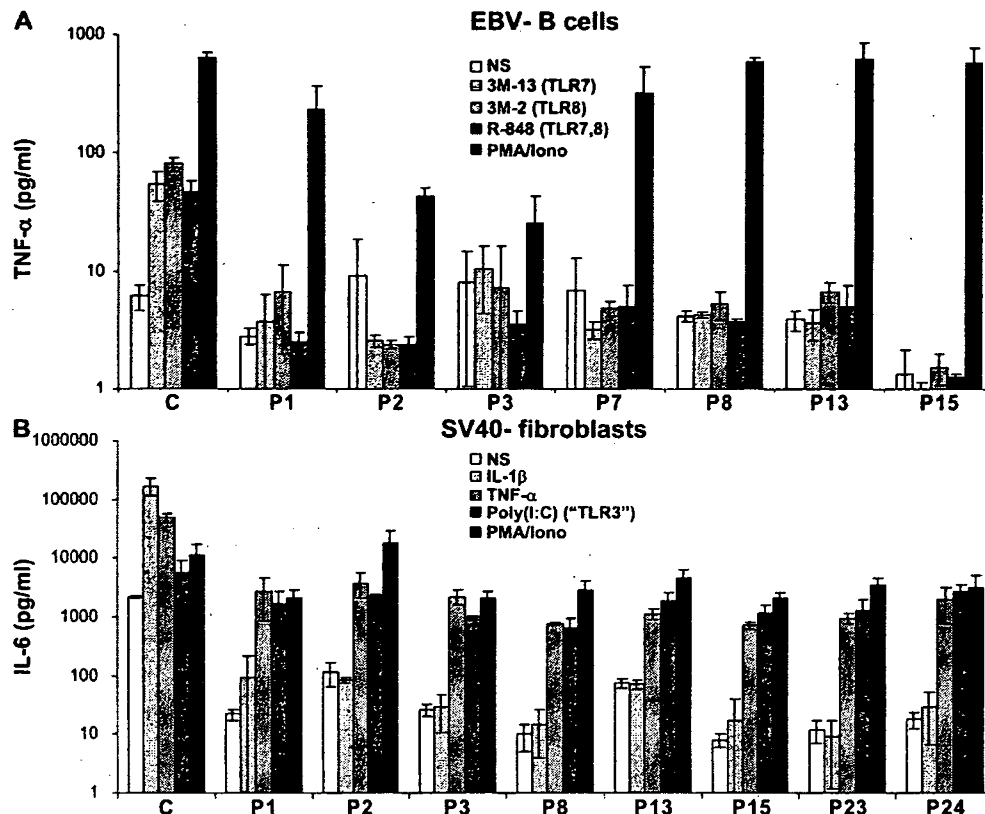

Figure 3. Impaired cellular responses to TIR agonists in IRAK-4–deficient cell lines. (A) TNF-α production by B-EBVs from a healthy control (C) and seven IRAK-4–deficient patients 24 h after stimulation with various TLR agonists and PMA/ionomycin. (B) IL-6 production by SV40-fibroblasts from a healthy control and eight IRAK-4–deficient patients after 24 h of stimulation with IL-1β, TNF-α, poly(I:C), and PMA/ionomycin. Mean values and SDs are shown for triplicates of a single experiment.

IL-6, IL-12, G-CSF, GM-CSF, and IFN-γ in vitro in response to all IL-1R and TLR agonists tested (1–9, 11, 12). We wondered whether the induction of other cytokines, chemokines, IFNs, and growth factors was also dependent on IRAK-4 after TLR stimulation. We therefore activated PBMCs from IRAK-4–deficient patients with Pam_3CSK_4 (TLR1/2), Pam_2CSK_4 (TLR2/6), poly(I:C) (a nonspecific TLR3 agonist), LPS (TLR4), flagellin (TLR5), 3M-13 (TLR7), 3M-2 (TLR8), R-848 (TLR7 and 8), and CpG (TLR9) for 24 h. We did not assess TLR10 responses, as there is no known agonist for this receptor (23). Cytokine secretion into the supernatant was assessed using a multiplex cytometry-based system. 11 out of the 25 cytokines assayed were induced and detectable after TLR stimulation in healthy controls. IRAK-4–deficient cells did not respond to seven out of nine agonists for all cytokines tested (Fig. 4). Upon activation with poly(I:C), the patients' PBMCs displayed induction of IL-12, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1β (MIP-1β) to levels similar to those in healthy controls, as well as some induction of IFN-inducible protein 10 (Fig. 4). However, the induction of IL-12 and MIP-1β was weak in both patients and healthy controls (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20070628/DC1). IL-7 induction was abolished in the patients, whereas other cytokines were not induced in controls. The patients' PBMCs showed detectable IL-8 and MIP-1β (an IFN-inducible cytokine) responses to LPS, but these responses were weaker than those of healthy controls (Fig. 4). The other cytokines were not induced in the patients. These data are reminiscent of our previous observation that IRAK-4–deficient PBMCs respond to poly(I:C) by producing IFN-α protein, and to poly(I:C) and LPS by producing IFN-β mRNA (13). However, whereas LPS responses can be specifically ascribed to TLR4, we recently showed, in TLR3-deficient patients, that the poly(I:C) responses of PBMCs are TLR3-independent (42). These data indicate a broad immunological impact of IRAK-4 deficiency, as the production of 11 key cytokines was completely impaired in response to all TLR agonists, with the exception of a couple of cytokines in response to poly(I:C) and LPS.

TLR responses of individual myeloid subsets

We then assessed the role of IRAK-4 in TLR signaling pathways in discrete leukocyte cell populations. Cell subsets other

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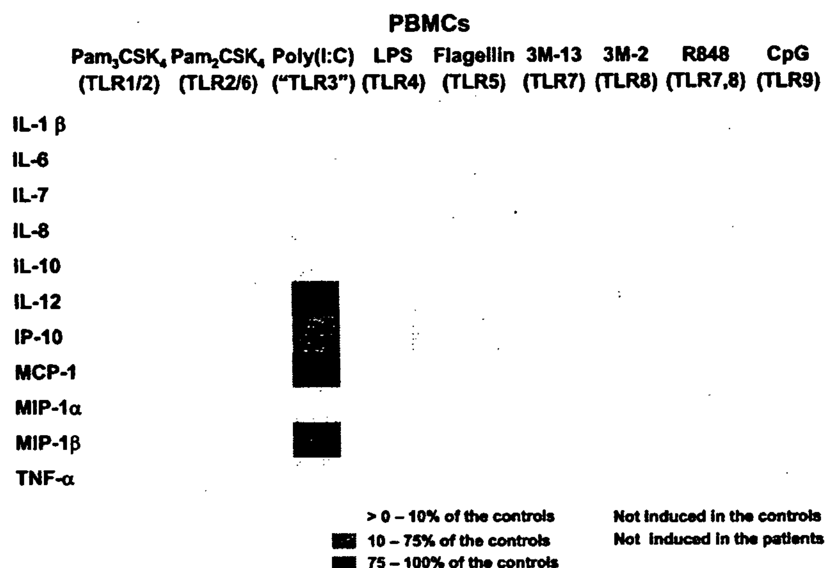

Figure 4. Multiple cytokine secretion in IRAK-4–deficient PBMCs. PBMCs from three healthy controls and three IRAK-4–deficient patients (P17, 18, and 22) were activated with various TLR agonists for 24 h. Cytokine levels are represented as ratios of the mean secretion observed in the three IRAK-4–deficient patients to that in three healthy controls. Cytokines represented in gray are not induced upon the stimulation of control PBMCs.

than granulocytes and DCs were purified by cell sorting (purity >99.5%). More than 95% of the granulocytes purified on Ficoll were $CD15^+$. The response of DCs (MDCs and PDCs) was tested in PBMCs. We assessed the CD62L shedding of granulocytes from four healthy controls and four IRAK-4–deficient patients after activation with Pam_3CSK_4, Pam_2CSK_4, LPS, flagellin, 3M-13, 3M-2, R-848, and TNF-α (10). The response to all TLR agonists was impaired in the granulocytes of all four patients tested (Fig. 5 A). $CD14^+$ monocytes from healthy controls responded to TLR1–8 agonists but not to TLR9 agonists. The monocytes of IRAK-4–deficient patients did not respond to these agonists, with the possible exception of very weak TNF-α production upon LPS stimulation (Fig. 5 B). Finally, we tested MDCs and PDCs by stimulating PBMCs from seven healthy donors and three IRAK-4–deficient patients with the TLR agonists Pam_3CSK_4, Pam_2CSK_4, poly(I:C), LPS, flagellin, 3M-13, 3M-2, R-848, and CpG for 3 h. We assessed TNF-α and MIP-1β production for MDCs (Lin-1^-, HLA-DR^+, and $CD123^{low}$) and PDCs (Lin-1^-, HLA-DR^+, and $CD123^{high}$) by intracellular staining. In healthy individuals, MDCs responded to all of the TLR agonists tested, except the TLR9 agonist, with the induction of TNF-α and MIP1-β. In contrast, only upon activation with poly(I:C) (nonspecific TLR3 agonist) and LPS (TLR4), did MDCs from the patients display normal levels of MIP1-β induction and some induction of TNF-α. PDCs from healthy individuals responded only to agonists of TLR7 and 9, whereas IRAK-4–deficient PDCs did not respond to any of the agonists tested (Fig. 5, C–F). As poly(I:C) activation in MDCs appears to be TLR3 independent (42), we further evaluated the production of TNF-α and the up-regulation of IFN-inducible surface-expressed CD40, CD80, and CD86 by in vitro MDDCs, which respond to poly(I:C) in a TLR3-dependent manner (42). MDDCs from healthy controls responded normally to the TLR agonists Pam_3CSK_4, Pam_2CSK_4, poly(I:C), LPS, flagellin, and 3M-2. In contrast, the patients' MDDCs did not respond to Pam_3CSK_4, Pam_2CSK_4, flagellin, and 3M-2. However, IRAK-4–deficient MDDCs showed a weak but not abolished TNF-α response and normal induction of CD40, CD80, and CD86 upon activation with poly(I:C) (TLR3). Normal induction of CD40, CD80, and CD86 was also observed upon activation with LPS (TLR4) (Fig. 5, G and H). These data indicate that the IRAK-4–deficient individual myeloid cell subsets tested displayed no response to most TLR agonists, with the exception of normal responses to poly(I:C) and LPS detected in MDCs for MIP-1β, an IFN type I–inducible cytokine, and in MDDCs for CD40, CD80, and CD86, which are induced by type I IFNs and TNF-α.

TLR responses of individual lymphoid subsets

We then tested the TLR responses of the B, T, and NK lymphoid cell subsets. The subsets were purified by cell sorting (purity >99.5%). $CD19^+$ B cells were activated by incubation with the TLR agonists Pam_3CSK_4, Pam_2CSK_4, poly(I:C), LPS, flagellin, 3M-13, 3M-2, R-848, and CpG for 24 h, and their response was measured by assessing IL-10 production. Highly purified control B cells showed a unique pattern of activation, with no response to agonists of TLR1/2, TLR2/6, TLR3, TLR4, TLR5, and TLR8, and only weak IL-10 production in response to TLR7, TLR7 and TLR8, and TLR9 agonists (Fig. 6 A and not depicted). In contrast, no response to these TLR agonists

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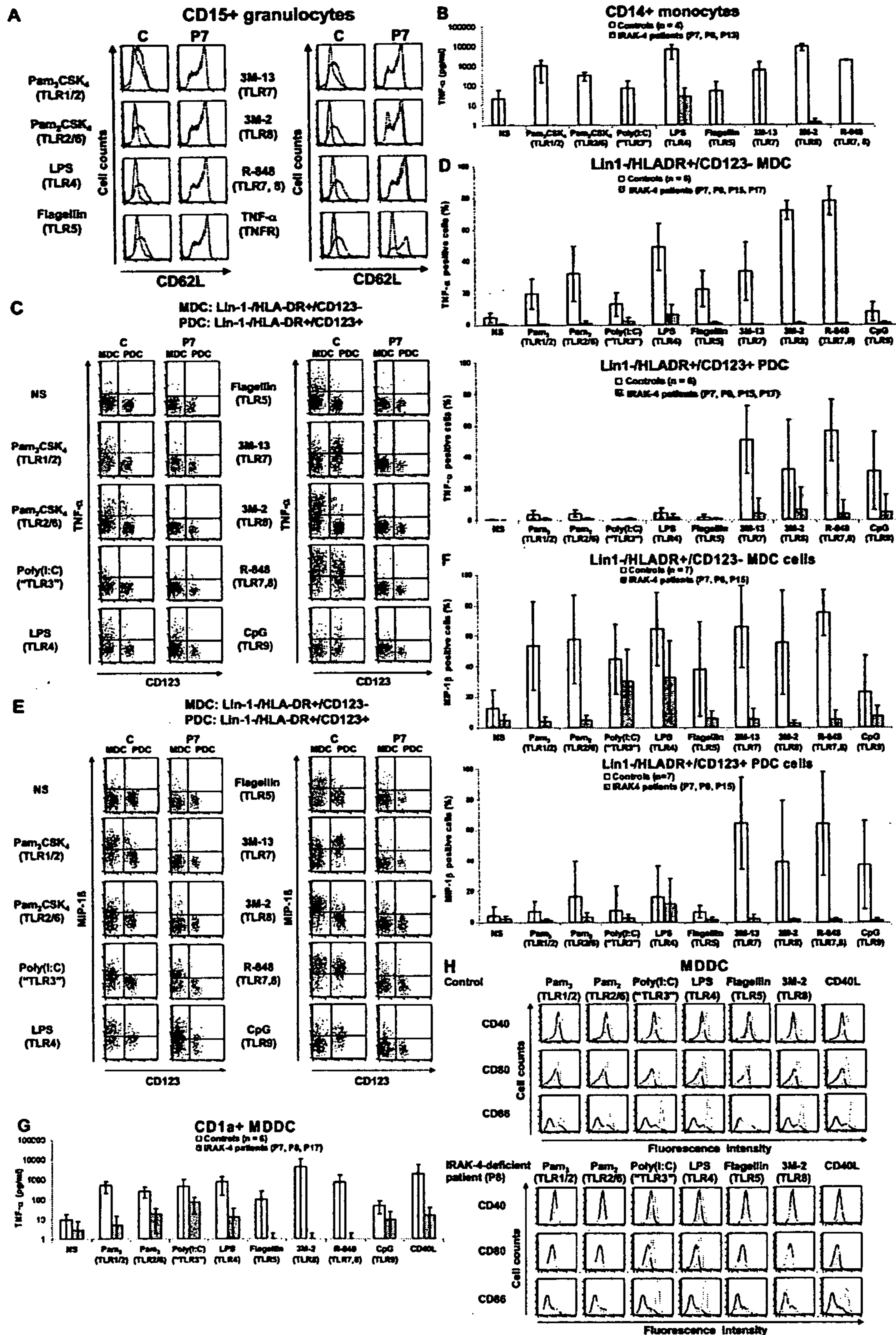

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was observed in the three IRAK-4–deficient patients tested (Fig. 6 A). Moreover, the response to TLR7 and 9, as measured by cell-surface expression of CD40, CD80, and CD86 after 3 d of incubation with IL-4 and various TLR agonists, was also impaired in the patients' B cells (Fig. 6 B) (13). $CD3^+$ T cells from healthy individuals were activated by Pam_3CSK_4, Pam_2CSK_4, poly(I:C), LPS, flagellin, 3M-13, 3M-2, R-848, and CpG. Control T cells displayed a weak but detectable response to Pam_3CSK_4 and flagellin in terms of IFN-γ production, whereas T cells from IRAK-4–deficient patients were not activated by any of the TLR agonists (Fig. 6 C). Finally, control NK cells were shown to respond to TLR3, 7, and TLR7 and 8 agonists in terms of IFN-γ production, but no response was observed in NK cells from IRAK-4–deficient patients (Fig. 6 D). NK cells respond to poly(I:C) through TLR3 (42), suggesting that at least some TLR3 pathways are IRAK-4 dependent. These data indicate that the three major blood lymphoid subsets require IRAK-4 for TLR responses, including TLR3 responses in NK cells.

Clinical features of IRAK-4 deficiency

In total, 28 IRAK-4–deficient patients from 18 families were studied, including the 7 patients (P21–27) from 5 families described in this study for the first time (Table I and Fig. 1). Most IRAK-4–deficient patients had had at least one Gram-positive bacterial infection: 22 out of the 28 (79%) had had invasive disease caused by *S. pneumoniae* (meningitis, septicemia, or arthritis), and 9 out of the 28 (32%) had suffered severe disease caused by *S. aureus* (meningitis, septicemia, or liver abscess; Table I). If we also take into account peripheral staphylococcal disease (cellulitis and subcutaneous abscess), 14 patients could be considered particularly susceptible to *S. aureus*. One patient (P20) had had no major infectious disease. This patient is 25 mo old and was diagnosed with IRAK-4 deficiency as a neonate. He was placed on IgG substitution and antibiotic prophylaxis shortly after birth. Seven patients also suffered from severe Gram-negative bacterial infections, which were invasive in four cases (*Shigella sonnei* and *P. aeruginosa*) and peripheral in four cases (*Escherichia coli*, *Serratia marcescens*, *Neisseria meningitidis*, and *P. aeruginosa*). As previously reported in a smaller series (13), no severe viral, fungal, or parasitic infections were observed in the patients. Most patients developed their first invasive infection before the age of 2 yr (20 out of 28; 71%), often before the age of 6 mo (9 out of 28; 32%) and in the neonatal period (4 out of 28; 14%), when maternal antibodies are still present. Remarkably, no invasive infection was documented in the six patients over the age of 14 yr (P2, 14 yr; P4, 24 yr; P7, 32 yr; P17 and 18, 27 yr; and P24, 16 yr), even in the absence of prophylaxis (P2, 4, 7, 17, and 18; $n = 5$; Fig. 7 A) (4, 6, 36). 12 patients died of invasive Gram-positive infections, all before the age of 8 yr and most before the age of 2 yr (Fig. 7 B). IRAK-4 deficiency is thus associated with a selective predisposition to pyogenic bacterial infections, mostly caused by Gram-positive bacteria (*S. pneumoniae* in particular and *S. aureus* to a lesser extent), and clinical status and outcome both improve with age. The detailed clinical features of IRAK-4 deficiency will be reported elsewhere (unpublished data).

DISCUSSION

The 28 patients reported in this study suffered from complete IRAK-4 deficiency. The patients had been exposed to an extremely diverse range of microorganisms, including many potential viral, bacterial, and fungal pathogens, as well as parasites (Tables S2 and S3, available at http://www.jem.org/cgi/content/full/jem.20070628/DC1). However, IRAK-4–deficient patients presented a strikingly narrow infectious phenotype (Table I), similar to the three patients initially reported (1). 27 patients suffered from invasive infectious disease, typically caused by Gram-positive *S. pneumoniae* ($n = 22$; 79%) and/or *S. aureus* ($n = 9$; 32%). Seven patients (25%) also presented severe infections with Gram-negative bacteria (*P. aeruginosa*, *N. meningitidis*, *S. sonnei*, and *S. marcescens*). 15 patients had peripheral infectious disease. When identified, the causal pathogens were *S. aureus*, *P. aeruginosa*, and *Streptococcus* species. The susceptibility of IRAK-4–deficient patients to *S. aureus* is consistent with that observed in IRAK-4– and MyD88-deficient mice (31, 43). MyD88-deficient mice are susceptible to *P. aeruginosa* (44) and, in some models, to *S. pneumoniae* (45, 46). Intriguingly, the 28 IRAK-4–deficient patients were not particularly susceptible to most other microorganisms, including common viruses (e.g., herpes viruses, enteroviruses, adenoviruses, and papillomaviruses), and widespread bacteria (e.g., *Listeria*, *Mycobacterium*, and Enterobacteriaceae), parasites (e.g., *Toxoplasma*), and fungi (e.g., *Cryptococcus*, *Pneumocystis*, *Candida*, and *Aspergillus*). As five of these patients have had

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Figure 5. Impaired responses to TLR agonists in IRAK-4–deficient individual myeloid subsets. (A) Cleavage of CD62 ligand (CD62L) at the surface of granulocytes from a healthy control and an IRAK-4–deficient patient (P7) after activation for 1 h with various TLR agonists and TNF-α. The black line shows CD62L expression on nonactivated granulocytes, and the red line shows CD62L expression after 1 h of activation with various agonists (induced CD62L shedding). One experiment representative of four (P7, 8, 13, and 15) is shown. (B) TNF-α secretion by $CD14^+$ monocytes after 24 h of activation with various TLR agonists. Mean values and SDs were calculated from four healthy controls and three IRAK-4–deficient patients. (C–F) Ex vivo MDC and PDC responses. PBMCs from healthy controls and IRAK-4–deficient patients were stimulated with various TLR agonists. In both subsets, responses were measured by staining for intracellular TNF-α (C) and MIP-1β (E). Mean values and SDs were calculated from six different controls and four IRAK-4–deficient patients for TNF-α (D), and from seven different controls and three IRAK-4–deficient patients for MIP-1β (F). (G) TNF-α secretion in vitro by MDDCs after 24 h of activation. Means and SDs were calculated from six different controls and three different IRAK-4–deficient patients. (H) Induction of CD40, CD80, and CD86 surface expression on MDDCs from a control (top) and an IRAK-4–deficient patient (bottom) after 24 h of stimulation with various TLR agonists. Black and green lines indicate the expression of CD40, CD80, and CD86 without and after stimulation, respectively. The experiment shown is representative of three independent experiments (also performed on patients P15 and 18). C, control.

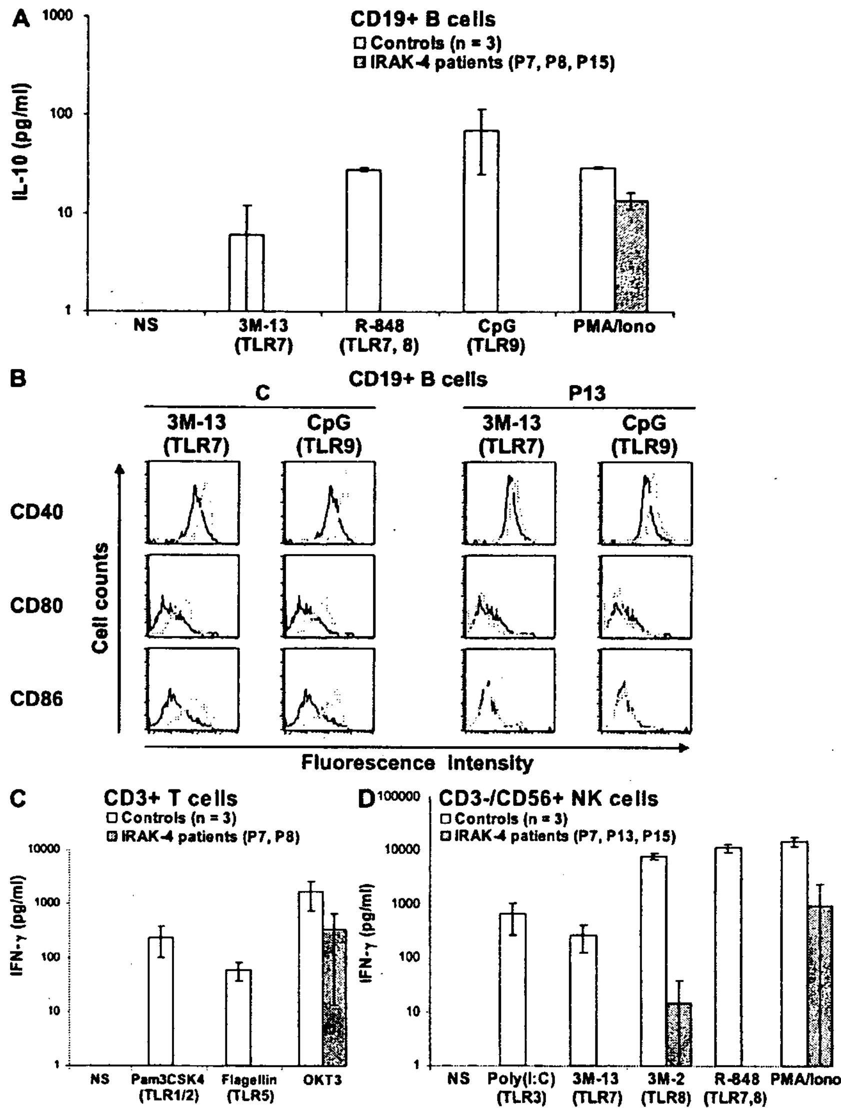

Figure 6. Lack of response to TLR agonists of individual IRAK-4–deficient lymphoid subsets. (A) IL-10 secretion by CD19$^+$ B cells after 24 h of activation with various TLR agonists and PMA/ionomycin. Mean values ± SD were calculated from the data obtained for three different controls and three IRAK-4-deficient patients. (B) Induction of CD40, CD80, and CD86 surface expression on CD19$^+$ B cells after activation for 72 h with 3M-13 and CpG. Black and green lines indicate the expression of CD40, CD80, and CD86 without and after stimulation, respectively. Data are representative of two independent experiments. (C) IFN-γ secretion by CD3$^+$ T cells after stimulation for 24 h with various TLR agonists and anti-CD3 (50 ng/ml OKT3) antibody in the presence of 100 U/ml IL-2 for 2 d. Mean values ± SD were calculated for three different controls and two IRAK-4-deficient patients. (D) IFN-γ secretion by CD3$^-$/CD56$^+$ NK cells after activation for 24 h with various TLR agonists and PMA/ionomycin. Mean values and SDs were calculated for three different controls and three IRAK-4-deficient patients.

no prophylaxis for 60 patient years (Fig. 7 B) (4, 6, 36), the resistance to most microbes observed is unlikely to be caused by the early death of some patients or to the prophylactic treatment of the survivors. Ascertainment bias cannot be excluded, but remains unlikely, as 10 affected relatives with causal mutations shared the case-definition clinical phenotype of index cases. In contrast, MyD88-deficient mice were found to be susceptible to mouse CMV (47), HSV-1(48), *Listeria monocytogenes* (49, 50), *Mycobacterium avium* (51), *Toxoplasma gondii* (52), *Cryptococcus neoformans* (53), *Candida albicans*, and *Aspergillus fumigatus* (54), among other relevant infections (37–39).

So why are the infectious phenotypes of MyD88/IRAK-4–deficient mice and IRAK-4–deficient humans so different? An overrepresentation of MyD88 deficiency with respect to IRAK-4 deficiency in mouse studies may be involved,

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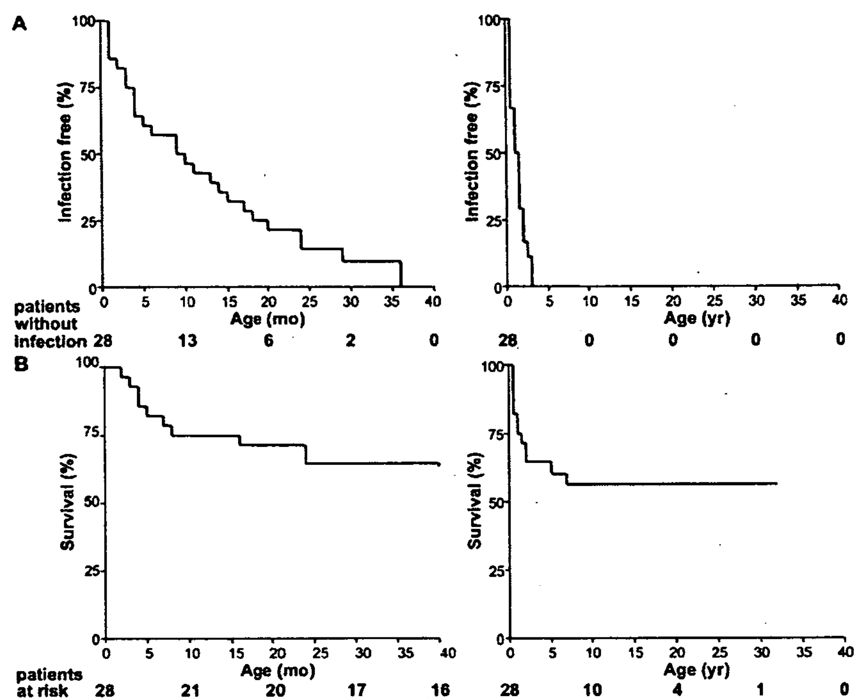

Figure 7. Epidemiological features of IRAK-4 deficiency. (A) Incidence of invasive infections in IRAK-4-deficient patients during the first 40 mo of life (left) and the first 40 yr of life (right). Invasive infections included meningitis, septicemia, and arthritis. (B) Survival curve of 28 IRAK-4-deficient patients during the first 40 mo of life (left) and the first 40 yr of life (right).

although IRAK-4– and MyD88-deficient mice, when infected by the same pathogens, are indistinguishable (31, 43). We provide an experimental demonstration in this paper that the occurrence of human-specific IRAK-4–independent TLR pathways is not involved. We show that IRAK-4–deficient PBMCs do not secrete any of 11 cytokines tested when stimulated with agonists of TLR1, 2, 5, 6, 7, 8, and 9. The TLR4 response was abolished for all but two cytokines, which were weakly induced. One of these two cytokines was the IFN-inducible MIP-1β, consistent with the IFN-β mRNA response to LPS in IRAK-4–deficient PBMCs (13). IRAK-4–deficient PBMCs also responded to poly(I:C), producing IFN-inducible monocyte chemoattractant protein 1 and IFN-inducible protein 10, as expected from the previously reported induction of IFN-α, -β, and -λ in IRAK-4–deficient PBMCs and fibroblasts (13). However, poly(I:C) activates PBMCs normally in patients with TLR3 deficiency (42), making it difficult to infer conclusions about TLR3 responses from the data for poly(I:C) stimulation. In any event, the MyD88- and IRAK-4–independent TLR3 and TLR4 pathways, present in mice, cannot account for humans being more resistant (13, 15). The "conventional" MyD88-dependent pathway downstream from TLRs appears to be strictly IRAK-4–dependent in humans; no detectable leakiness can apparently account for the narrow infectious phenotype. We cannot, however, exclude the possibility that other TLR-inducible genes may be IRAK-4 independent.

We further excluded the possibility that human IRAK-4 deficiency may be milder than mouse MyD88/IRAK-4 deficiency owing to the occurrence of human-specific IRAK-4–independent TLR pathways in discrete leukocyte subsets, as suggested by the normal induction of both IL-6 and IFN-β/λ in IRAK-4–deficient fibroblasts (13). We showed that IRAK-4 deficiency impaired the TLR responses of all lymphoid and myeloid leukocyte subsets tested ex vivo, including granulocytes, monocytes, PDCs, MDCs, NK, T, and B cells. With the exception of the induction of IFN-inducible MIP-1β production in MDCs in response to poly(I:C) and LPS (Fig. 5, E and F), there was no detectable TLR response in individual subsets. The LPS response is TLR4 dependent, whereas the poly(I:C) response in MDCs appears to be TLR3 independent (42). Even IRAK-4–deficient NK cells did not respond to poly(I:C), suggesting that responses to poly(I:C) in NK cells are largely TLR3- (42) and IRAK-4–dependent. Moreover, MDDCs generated in vitro did not respond to TLR agonists, with the exception of poly(I:C) and LPS. The poly(I:C)-triggered induction of TNF-α, CD40, CD80, and CD86 in MDDCs was IRAK-4 independent (Fig. 5, G and H) and seemed to be TLR3 dependent (42). These data extend previous findings (1, 13) and show that human IRAK-4 plays a nonredundant role in the conventional TLR signaling pathway in at least seven major leukocyte subsets. In contrast, IRAK-4 may be dispensable for the "alternative," TRIF-dependent pathways downstream from TLR3 (for IFNs and other cytokines) and

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