abscesses affected the brain (3 episodes), peritoneum (8 episodes), liver (4 episodes), and muscles (2 episodes: subfascial calf and psoas abscesses).

There were 33 reported episodes of InvBD in 12 MyD88-deficient patients (n = 2.75 episodes per patient; range, 1–7), including meningitis (17 episodes, 51.5% of all invasive episodes), sepsis (4 episodes, 12.1%), arthritis (6 episodes, 18.2%), osteomyelitis (2 episodes, 6.1%), and deep inner organ/tissue abscesses (4 episodes, 12.1%).

Five IRAK-4-deficient patients never developed InvBD, 4 of whom were diagnosed at birth and remained asymptomatic on prophylactic treatment (Figure 2; Table 1). The remaining patient without InvBD was diagnosed at the age of 2 years following an episode of *Staph. aureus* adenitis (N-P23) and received prophylactic treatment from that time to the end of follow-up. All the MyD88-deficient patients reported have presented InvBD (Figure 5; Table 1). Neurologic complications secondary to meningitis and brain abscesses occurred in 7 IRAK-4-deficient patients; 5 of them developed secondary deafness (K-P17, Q-P27, R-P28, W-P35, X-P37), and 2 other patients developed hemiplegia (B-P2) or developmental delay (M-P22). Three MyD88-deficient patients (c-P3, c-P4, e-P9) developed secondary deafness. The overall frequency and the sites of InvBD were found to be indistinguishable in IRAK-4-deficient and MyD88-deficient patients.

NonInvasive Bacterial Infections

Noninvasive bacterial disease (NInvBD) most frequently presented as skin infections, such as recurrent localized cellulitis, furunculosis, and folliculitis, often prompting intravenous and prolonged antibiotic treatment (in 21 of 48 IRAK-4-deficient and 3 MyD88-deficient patients) (Figure 6). IRAK-4-deficient patients also presented with adenitis (14 patients), omphalitis (6 patients), maxillary sinusitis (6 patients), tonsillar abscesses (4 patients), necrotizing epiglotitis (1 patient), necrotizing pharyngitis (1 patient), necrotizing palate infection (1 patient), recurrent otitis media (12 patients), and orbital cellulitis or endophthalmitis (6 patients). MyD88-deficient patients developed adenitis (5 patients), sinusitis (2 patients, a-P1 and c-P3), recurrent otitis media (2 patients), gingivitis and periodontal disease (1 patient, c-P3). Intriguingly, only 21 episodes of pneumonia were reported, in only 9 IRAK4-deficient patients and 2 MyD88-deficient patients. There were no episodes of acute bronchitis and no chronic bronchopulmonary disease. Acute upper urinary tract infections were found in only 2 IRAK-4-deficient patients and 1 MyD88-deficient patient. Most NInvBD in MyD88-deficient and IRAK-4-deficient patients affected the skin and the upper respiratory tract—sites at which necrotizing infections are particularly common.

Documented Bacterial Infections

In both IRAK-4 and MyD88 deficiency, *Str. pneumoniae*, *Staph. aureus*, and *P. aeruginosa* were, by far, the most commonly isolated pathogens causing InvBD and NInvBD (Figure 7; Table 1). In IRAK-4-deficient patients, *Str. pneumoniae* accounted for 40.1% (67/167), *Staph. aureus* for 25.1% (42/167), and *P. aeruginosa* for 19.7% (33/167) of all documented bacterial infections (a total of 84.9%). *Str. pneumoniae* was involved in 54.3% (57/105) of InvBD episodes, whereas *Staph. aureus* and *P. aeruginosa* were found in 14.3% (15/105) and 18% (19/105) of such episodes, respectively, accounting together for 87% of all cases of InvBD. The other bacteria causing invasive disease were *Streptococcus* species, *Shigella sonnei*, *Neisseria meningitidis*, *H. influenzae* type b, and *Clostridium septicum* (Table 1). In cases of NInvBD, the principal bacterium isolated was *Staph. aureus*, which was implicated in 43.5% (27/62) of documented episodes of NInvBD, whereas *P. aeruginosa* and *Str. pneumoniae* were found in 22.6% (14/62) and 16.1% (10/62), respectively. These 3 bacteria altogether accounting for 82% of all episodes of NInvBD.

In patients with MyD88 deficiency, *Str. pneumoniae* accounted for 37.5% (18/48), *Staph. aureus* for 31.2% (15/48), and *P. aeruginosa* for 12.5% (6/48) of all bacterial infections (81%) (Figure 7). *Str. pneumoniae* caused InvBD in 45.5% of cases (15/33), whereas *Staph. aureus* and *P. aeruginosa* were involved in 21.2% (7/33) and 12.1% (4/33) of the episodes, respectively (78.8% of all cases of InvBD). The other pathogens identified during invasive infections were β-hemolytic *Streptococci*, *Salmonella enteritidis*, *H. influenzae* type e, and

P36	P13	P37	P1	P15	P28	P34	P8	P3	Normal Values	P31	P24	P2	P17	P18	P7	Normal Values
3 yr	4 yr	4 yr	5 yr	5 yr	5 yr	5 yr	6 yr	7 yr	(3–7 yr)	11 yr	13 yr	14 yr	27 yr	27 yr	32 yr	(11 yr-adult)
11.2	13.6	9.29	11.7	10.3	18.99	11.1	13.6		(5.49–11.54)	12.8	7.6	14.4	15	13	16.7	(6.55–12.78)
8.82	10.2		6.85	6.88	8.44		8.41		(>4)	10.6	7.16	8.62				(>4)
0.65	2.63		0.4	1.18	5.37		3.12		(>0.40)	2.5	0.95	0.71				(>0.60)
0.36	0.13		0.19	0.45	0.29		0.34		(>0.16)	0.72	0.38	0.47				(>0.17)
0.29	1.85		1.41	0.5	4.89		3.54		(<1)	0.65	0.06	1.46				(<1)
1.19	0.64	0.57	0.63	1.31	1.51	0.6	2.42		(0.41–1.57)	1.02	0.49	0.88	0.3	0.6	1.1	(0.70–3.44)
1.59	1.65	0.85	0.72	0.50	0.96	3.5	0.52		(0.54–1.55)	1.38	1.91	0.64	1.3	1.5	1.9	(0.50–2.09)
6	977	106	17,400	356			187		(<60)	180	36.6	11	255	96.5	400	(<150)
0.58	>10			0.06	0.26	0.47	0.59	1,81	(>0.1 IU/mL)		0.47	0.47	0.34	0.46	0.06	(>0.1 IU/mL)
				>40					(>40)			40				(>40)
0.55			2.04	0.002			0.10	0.88	(>0.1 IU/mL)		0.72				0,18	(>0.1 IU/mL)
	0.2		1.9	>0.6	<0.3	>0.3	<0,3		(>0.3 µg/mL)		3.59	<0.3	<0.3	<0.3	>0.3	(>0.3 µg/mL)
>1	5.1	0.84	>1		1.98	12.3	>1	>1	(>0.15 µg/mL)			>1			1.36	(>0.15 µg/mL)
	1/8	1/1	1/128	1/16			1/2		(>1/16)		1/32		1/16	1/16	1/2	(>1/16)

TABLE 5. Immunologic Investigation: Ig Levels and Humoral Responses to Recall Antigens and to Glycans in MyD88–Deficient Patients*

Patients	P5	P10	Normal Values	P2	P6	P9	Normal Values	P8	P4	Normal Values	P3	Normal Values
(age)	8 mo	15 mo	(6–15 mo)	2 yr	2.5 yr	3 yr	(2–4 yr)	7 yr	8 yr	(5–8 yr)	15 yr	(14 yr–adult)
Serum Ig (g/L)												
IgG	2.23	6.2	(2.35–6.23)	12.4	13.6	29.2	(4.82–8.96)	12.5	8.31	(5.49–11.54)	12.5	(6.55–12.78)
IgG1		5.0	NA	10.3	10.36	20.4	(>4)	8.0	6.61	(>4)	9.8	(>4)
IgG2		0.88	NA	0.148	2.52	5.19	(>0.3)	3.2	0.96	(>0.40)	0.85	(>0.6)
IgG3		0.78	NA	0.59	0.3	1.7	(>0.15)	0.47	0.24	(>0.16)	0.56	(>0.17)
IgG4		0.07	NA	0.44	0.4	3.51	(<1)	3.7	0.1	(<1)	0.84	(<1)
IgA	0.36	0.52	(0.12–0.62)	0.5	0.32	1.6	(0.33–1.22)	1.32	1.21	(0.41–1.57)	1.36	(0.70–3.44)
IgM	0.41	0.93	(0.34–1.1)	1.59	0.61	1.24	(0.50–1.53)	0.29	0.9	(0.54–1.55)	0.69	(0.50–2.09)
IgE (U/mL)	53		(<30)	24.5	115	34	(<40)		199	(<60)	10.4	(<150)
Specific antibodies												
Antitetanus			(>0.1 IU/mL)			0.9	(>0.1 IU/mL)		1.3	(>0.1 IU/mL)		(>0.1 IU/mL)
S. pneumoniae		0.9	(>0.3 µg/mL)	17.96		11.5	(>0.3 µg/mL)		2.74	(>0.3 µg/mL)	3.85	(>0.3 µg/mL)
H. influenzae b			(>0.15 µg/mL)			3.4	(>0.15 µg/mL)			(>0.15 µg/mL)		(>0.15 µg/mL)
Allohemagglutinin			NA	1/4			(>1/8)		1/16		1/16	(>1/16)

*Serum immunoglobulin levels and titers for specific antibodies. Age-specific normal values are shown in parentheses.

Moraxella catarrhalis. In cases of NInvBD, the principal bacterium isolated was *Staph. aureus*, which was implicated in 53.3% (8/15) of NInvBD episodes, whereas *Str. pneumoniae* was found in 20% (3/15) and *P. aeruginosa* in 13.3% (2/15) of NInvBD episodes. These 3 bacteria accounting altogether for 86% of all cases of NInvBD.

In summary, in both IRAK-4 and MyD88 deficiencies, *Str. pneumoniae*, *Staph. aureus*, and *P. aeruginosa* were by far the most commonly isolated pathogens causing InvBD (52.2%, 15.9%, and 16.7% of cases, respectively), and *Staph. aureus* was by far the most commonly isolated pathogen causing NInvBD (45.5%) (Figure 7).

Other Infections

Among infections caused by agents other than pyogenic bacteria, there were no severe mycobacterial, viral, parasitic, and fungal diseases. One IRAK-4-deficient patient (K-P18) had a *Mycobacterium avium* lung infection and otitis at the age of 15 years. Nine patients (8 IRAK-4-deficient patients and 1 MyD88-deficient patient) received Bacille Calmette-Guérin (BCG) vaccination without adverse effect. One IRAK-4-deficient patient (R-P28) had *Staph. aureus* meningitis at the age of 6 years, and *Enterovirus* was isolated from the cerebral spinal fluid by polymerase chain reaction. Another IRAK-4-deficient patient (C-P3) had an episode of diarrhea caused by *Enterovirus* at the age of 7 years. One MyD88-deficient patient (a-P1) experienced 2 hospital-acquired episodes of diarrhea caused by adenovirus and rotavirus, with both infections following a normal course during the first year of life. One MyD88-deficient patient (d-P5) had 3 episodes of respiratory syncytial virus bronchilitis at 2, 3, and 4 months of age, with a spontaneous favorable outcome. One IRAK-4-deficient patient (T-P31) developed localized warts at the age of 16 years. One MyD88-deficient patient (d-P6) developed chickenpox 10 days after varicella zoster virus vaccination. Several IRAK-4- and MyD88-deficient patients had humoral responses to viruses and *Toxoplasma gondii* without abnormal clinical manifestations (Table 7). Two IRAK-4-deficient patients (C-P3, W-P36) and 2 MyD88-deficient patients (c-P4, e-P9) had oral thrush, even in the absence of antibiotic treatment. Finally, *Curvularia* species were isolated from the maxillary sinus of 1 IRAK-4-deficient patient (C-P3) living in the southern United States.

In conclusion, it is noteworthy that IRAK-4-deficient and MyD88-deficient patients were not particularly susceptible to most other microorganisms, including common viruses (for example, herpes viruses, enteroviruses, adenoviruses, and papillomaviruses), and widespread bacteria (for example, *Listeria* and *Mycobacterium*), parasites (for example, *Toxoplasma*), and fungi (for example, *Cryptococcus, Pneumocystis, Candida,* and *Aspergillus*).

Patient Outcome

Most IRAK-4-deficient patients had their first bacterial infection early in life, before the age of 2 years in 87.5% (n = 42) of cases. The first InvBD occurred before the age of 2 years in 79.2% (n = 38), and the first NInvBD in 48% (n = 23) of these patients. The first bacterial infection occurred before the age of 6 months in 54% (n = 26) of IRAK-4-deficient patients. The first InvBD occurred before the age of 6 months in 35.4% (n = 17), and the first NInvBD in 37.5% (n = 18) of these patients. The first bacterial infection even occurred during the neonatal period in 31.2% (n = 15) of IRAK-4-deficient patients. The first InvBD occurred during the neonatal period in 14.5% (n = 7) and the first NInvBD in 27% (n = 13) of these patients (5 patients had both InvBD and NInvBD in the neonatal period) (Figures 8 and 9).

TABLE 6. Immunologic Investigation Summary

	IRAK-4-Deficient Patients	MyD88-Deficient Patients
T lymphocytes subset		
Normal pts/tested pts (%)	24/24 (100)	6/6 (100)
B lymphocytes subset		
Normal pts/tested pts (%)	23/23 (100)	7/7 (100)
NK lymphocytes subset		
Normal pts/tested pts (%)	19/19 (100)	6/6 (100)
T cell proliferation		
Normal pts/tested pts (%)	12/12 (100)	3/3 (100)
IgG levels		
Normal pts/tested pts (%)	15/28 (53.6)	3/8 (37.5)
Pts with increased level/tested pts (%)	12/28 (42.9)	4/8 (50)
Pts with decreased level/tested pts (%)	1/28 (3.6)	1/8 (12.5)
IgG1,2,3 levels		
Normal pts/tested pts (%)	13/13 (100)	7/7 (100)
IgG4 levels		
Normal pts/tested pts (%)	8/13 (61.5)	5/7 (71.4)
Pts with increased level/tested pts (%)	5/13 (38.5)	2/7 (28.6)
IgA levels		
Normal pts/tested pts (%)	25/28 (89.3)	7/8 (87.5)
Pts with decreased level/tested pts (%)	3/28 (10.7)	—
Pts with decreased level/tested pts (%)	—	1/8 (12.5)
IgM levels		
Normal pts/tested pts (%)	26/28 (92.9)	6/8 (75)
Pts with increased level/tested pts (%)	2/28 (7.1)	1/8 (12.5)
Pts with decreased level/tested pts (%)	—	1/8 (12.5)
IgE levels		
Normal pts/tested pts (%)	6/20 (30)	3/6 (50)
Pts with increased level/tested pts (%)	14/20 (70)	3/6 (50)
Specific Ab to protein antigens (tetanus, diphtheria, or polio)		
Normal pts/tested pts (%)	17/17 (100)	2/2 (100)
Ab against *H. influenzae*		
Normal pts/tested pts (%)	14/14 (100)	1/1 (100)
Ab against *S. pneumoniae*		
Normal pts/tested pts (%)	6/13 (46.2)	5/5 (100)
Pts with abnormal response/tested pts (%)	7/13 (53.8)	
Ab production after immunization with PNCV23		
Normal pts/tested pts (%)	4/9 (44.4)	
Pts with abnormal response/tested pts (%)	5/9 (55.6)	
Ab production after immunization with PNCV23+PCV7		
Normal pts/tested pts (%)	2/3 (66.7)	
Pts with abnormal response/tested pts (%)	1/3 (33.3)	
Ab production after immunization with PCV7		
Normal pts/tested pts (%)	1/1 (100)	
Allohemagglutinin		
Normal pts/tested pts (%)	7/10 (70)	3/3 (100)
Pts with decreased level/tested pts (%)	3/10 (30)	

Abbreviations: Ab = antibody, PCV7 = 7 valent conjugate vaccine, PNCV23 = 23 valent nonconjugate vaccine, pts = patients.

Similarly, bacterial infections occurred early in most MyD88-deficient patients, before the age of 2 years in 91.7% (n = 11) of these patients. The first InvBD occurred before the age of 2 years in 50% (n = 6), and the first NInvBD in 66.7% (n = 8) of these patients. The first bacterial infection occurred before the age of 6 months in 91.7% (n = 11) of MyD88-deficient patients. The first InvBD occurred before the age of 6 months in 50% (n = 6), and the first NInvBD in 66.7% (n = 8) of the cases. The first bacterial infection occurred in the neonatal period in 33.3% (n = 4) of MyD88-deficient patients. The first InvBD occurred during the neonatal period in 16.7% (n = 2), and NInvBD in 16.7% (n = 2) of these patients (Figures 8 and 9).

IRAK-4-deficient patients presented no InvBD from the age of 14 years on (a total of 10 patients, aged 14, 15, 17, 18, 19, 27,

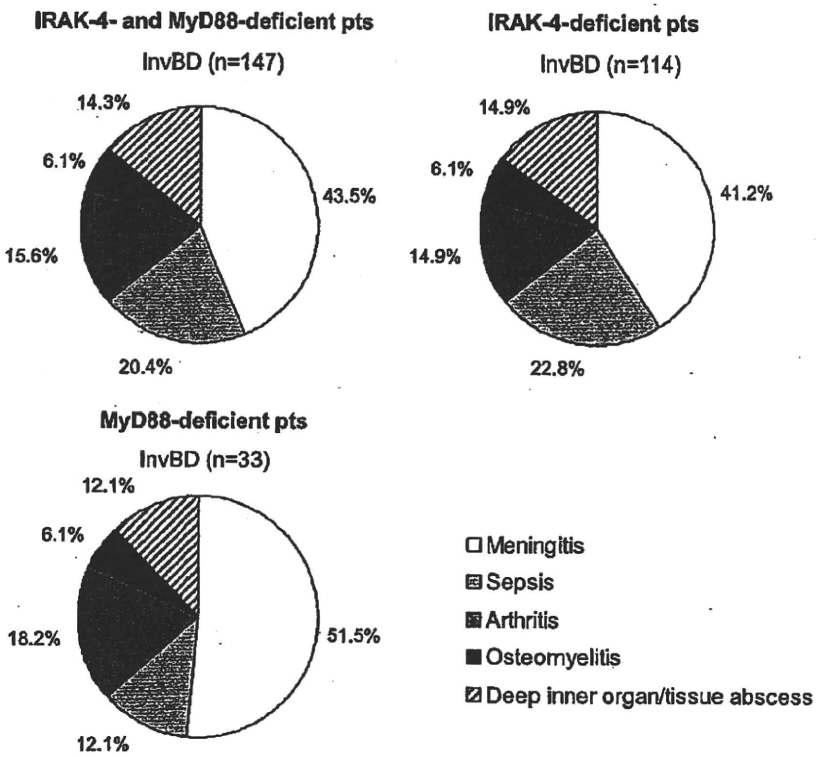

FIGURE 5. Invasive bacterial infections (episodes): in all patients, in IRAK-4-deficient patients, and in MyD88-deficient patients.

30, and 35 years), but the oldest patient, who was aged 35 years, still suffered from occasional skin infections at last follow-up (Figures 8 and 9). MyD88-deficient patients presented no InvBD from the age of 11 years on (2 patients aged 11 and 17 years), but the oldest patient, aged 17 years, still suffered from NInvBD at last follow-up. InvBD was recurrent (2–10 episodes) in 33 of the IRAK-4-deficient patients. In 3 IRAK-4-deficient patients, 2–3 recurrences of invasive pneumococcal disease due to the same serotype (6A, 14, or 19F) were identified at intervals of 1–24 months. InvBD was recurrent (2–7 episodes) in 5 of the MyD88-deficient patients. There were 114 reported episodes of InvBD in 48 IRAK-4-deficient patients (n = 2.38 episodes per patient; range, 0–10), and 33 reported episodes of InvBD in 12 MyD88-deficient patients (n = 2.75 episodes per patient; range, 1–7). Finally, 24 patients died of InvBD (18/48 IRAK-4, 6/12 MyD88), all before the age of 8 years, and most before the age

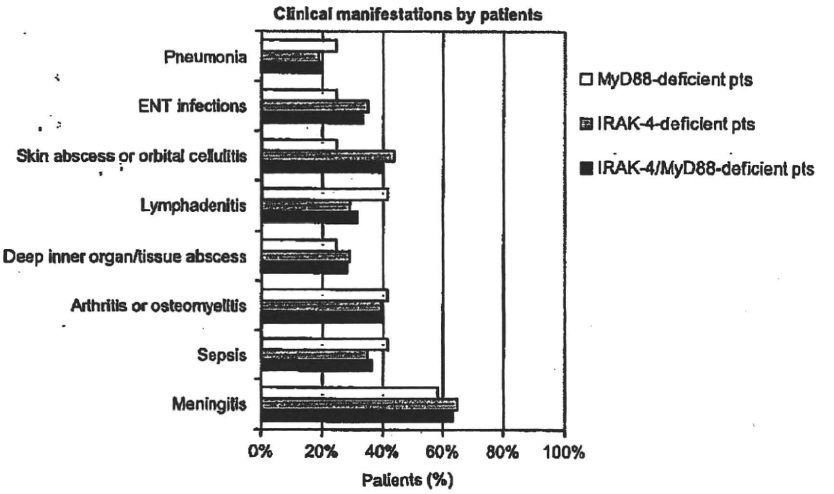

FIGURE 6. Percentage of clinical manifestations found in each patient: in MyD88-deficient patients, in IRAK-4-deficient patients, and in all patients. (ENT = ear, nose, and throat.)

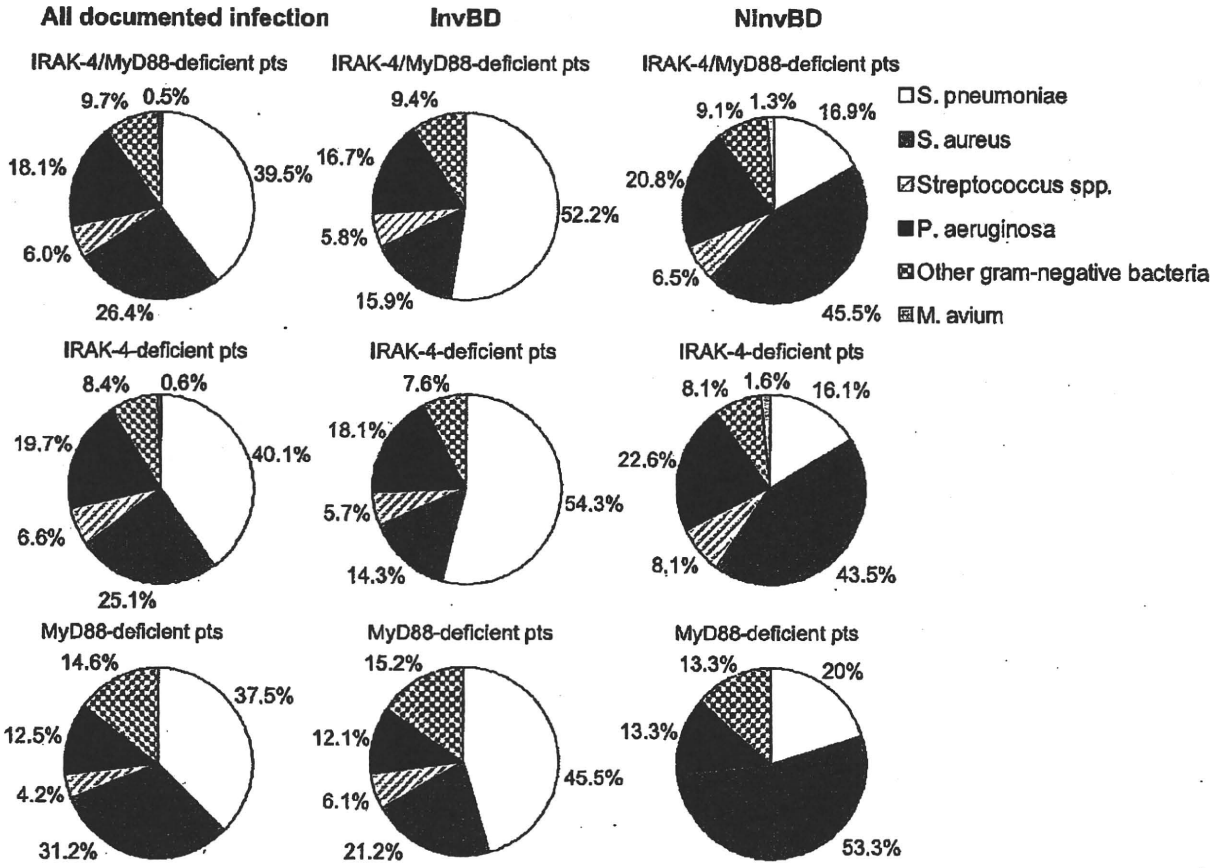

FIGURE 7. Overview of pathogens isolated during bacterial infections of IRAK-4-deficient and MyD88-deficient patients. **Left column,** overview of all pathogens isolated (all documented infection). In IRAK-4-deficient patients: other *Streptococcus* species (*Str. agalactiae, Str. equis, Str. intermedius, Str. milleri, Str. pyogenes,* and *Str. parasanguis*), other gram-negative bacteria (*Shigella sonnei, Neisseria meningitidis, Serratia marcesens, Moraxella catarrhalis, Clostridium septicum, Haemophilus influenzae* type b, *Citrobacter freundii,* and *Escherichia coli*), and *Mycobacterium avium*. In MyD88-deficient patients: other *Streptococcus* species (β-hemolytic *Streptococci*) and other gram-negative bacteria (*Salmonella enteritidis, Haemophilus influnzae* type e, *Moraxella catarrhalis, Klebsiella pneumoniae,* and *E. coli*). **Center column,** pathogens isolated during invasive bacterial infections (InvBD) (meningitis, sepsis, arthritis, osteomyelitis, and deep abscesses). In IRAK-4-deficient patients: other *Streptococcus* species (*Str. agalactiae, Str. milleri, Str. pyogenes,* and *Str. parasanguis*) and other gram-negative bacteria (*Shigella sonnei, N. meningitidis, Serratia marcesens, H. influenzae* type b and *C. septicum*). In MyD88-deficient patients: other *Streptococcus* species (β-hemolytic *Streptococci*) and other gram-negative bacteria (*Salmonella enteritidis, H. influenzae* type e, and *Moraxella catarrhalis*). **Right column,** pathogens isolated during noninvasive bacterial infections (NinvBD). In IRAK-4-deficient patients: other *Streptococcus* species (*Str. equis, Str. intermedius, Str. pyogenes*) and other gram-negative bacteria (*Serratia marcesens, Moraxella catarrhalis, C. septicum, Citrobacter freundii,* and *E. coli*), and *M. avium*. In MyD88-deficient patients: other *Streptococcus* species (β-hemolytic *Streptococci*) and other gram-negative bacteria (*K. pneumoniae* and *E. coli*).

of 2 years (n = 17) (Figure 10; Table 1). Sixteen of these patients died of invasive pneumococcal disease (11 IRAK-4-deficient and 5 MyD88-deficient patients).

Inflammatory Response

Impaired ability to mount inflammation during invasive infections has been previously described in isolated case reports and smaller series.[12,18,25,46] In the current study we evaluated temperature, C-reactive protein (CRP) levels, total leukocyte counts, and neutrophil counts in invasive infections during 3 periods of life that are known to have different levels of inflammatory responses: the neonatal period (day 1 to day 28), infancy (day 29 to 1 year), and childhood (children aged >1 year). In analyses carried out on admission to the hospital, we often observed inflammatory signs within the normal range, despite infection (Figures 11–13; Tables 8 and 9). Little (n = 3) or no (n = 2) increase in body temperature above 37°C was observed in neonates with IRAK4-deficiency. By contrast, a significant increase in CRP concentration (>10 mg/L) was observed in all neonates with IRAK-4 deficiency and InvBD. Counts of total leukocytes and of neutrophils remained low despite InvBD; none of the neonates showed neutrophil counts above the 95th percentile adjusted for age.[29] Initial temperature on admission was below 38°C in 10 of the 23 cases of InvBD in infants and in 22 of the 44 cases of InvBD in children admitted. Similarly, initial CRP concentration was below 10 mg/L in 12 of 23 cases of InvBD in infancy and in 16 of 36 cases of InvBD in childhood. Despite the presence of InvBD, total leukocyte counts remained below 14,000/μL in 21 of 35 episodes in infancy and in 46 of 52 episodes in childhood. One frequently documented abnormality was a neutrophil count below 6000/μL, observed in 20 of 26 episodes in infancy and 30 of 47 InvBD episodes in childhood.

TABLE 7. Humoral Responses to Viruses and *Toxoplasma gondii*

	IRAK-4-Deficient Patients (Positive pts/Tested pts)	MyD88-Deficient Patients (Positive pts/Tested pts)
Herpes simplex virus	0/8	2/4
Varicella zoster virus	5/9	2/3
Cytomegalovirus	2/9	3/4
Epstein-Barr virus	4/8	3/5
HHV6	6/6	Not done
HHV8	0/2	Not done
Parvovirus B 19	2/6	Not done
Rubella	5/6	4/4
Measles	Not done	3/4
Mumps	5/6	2/3
Coxsackie virus B1,2,3,4,6	6/7	Not done
RSV	6/6	Not done
Human metapneumovirus	5/6	Not done
Rotavirus	Not done	1/1
Adenovirus	Not done	1/1
HIV	0/3	Not done
VDRL	0/1	Not done
Toxoplasma	0/3	1/3

Abbreviations: See previous tables. HHV = human herpes virus, HIV = human immunodeficiency virus, VDRL = Venereal Disease Research Laboratory test.

Thus, both MyD88 and IRAK-4 deficiencies confer a predisposition to severe InvBD impairment of the ability to increase plasma CRP concentrations and mount fever. However, patients with IRAK-4 and MyD88 deficiency and InvBD may also present with high temperature and high levels of CRP, total leukocytes, and neutrophils (Figures 11–13). Pus formation was observed in the liver, joints, lymph nodes, saliva glands, and in the meninges, as well as in skin infections. Finally, separation of the umbilical cord later than 28 days after birth was observed in 10 IRAK-4-deficient patients.

Prophylaxis of Infections

Thirty-six patients with IRAK-4 deficiency or MyD88 deficiency received prophylaxis following diagnosis of the corresponding primary immunodeficiency, a diagnosis that occurred after 1 episode of InvBD in 30 patients (24 IRAK-4-deficient and 6 MyD88-deficient) and before any InvBD episode in 6 IRAK-4-deficient patients. Prophylactic treatment was discontinued in 7 (6 IRAK-4-deficient and 1 MyD88-deficient) of the 11 patients who reached the age of 14 years, and was continued in all others.

Preventive treatment included antibiotic prophylaxis (oral penicillin and/or cotrimoxazole in most cases (Table 10) in 28 IRAK-4-deficient and 6 MyD88-deficient patients, and empirical intravenous or subcutaneous IgG injections (400 mg/kg every 3 wk) in 15 IRAK-4-deficient and 4 MyD88-deficient patients. Patients were also immunized with *Str. pneumoniae* conjugated vaccine only (7/48 IRAK-4-deficient patients, 3/12 MyD88-deficient patients), nonconjugated vaccine only (8/48 IRAK-4-deficient patients, 1/12 MyD88-deficient patients), or both (9/48 IRAK-4-deficient patients, 3/12 MyD88-deficient patients); *H. influenzae* conjugated vaccine (21/48 IRAK-4-deficient patients, 8/12 MyD88-deficient patients); and *N. meningitidis* conjugated

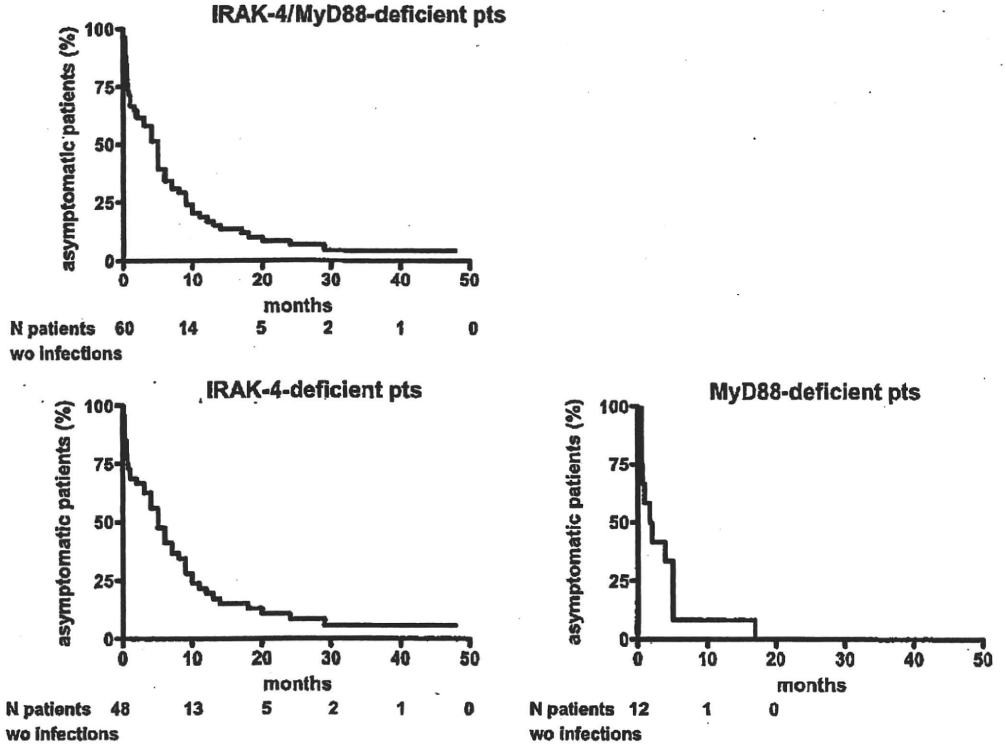

FIGURE 8. Epidemiologic features of IRAK-4 and MyD88 deficiency. Incidence of first bacterial infection in IRAK-4-deficient and MyD88-deficient patients during the first 50 months of life. (wo = without, pts = patients.)

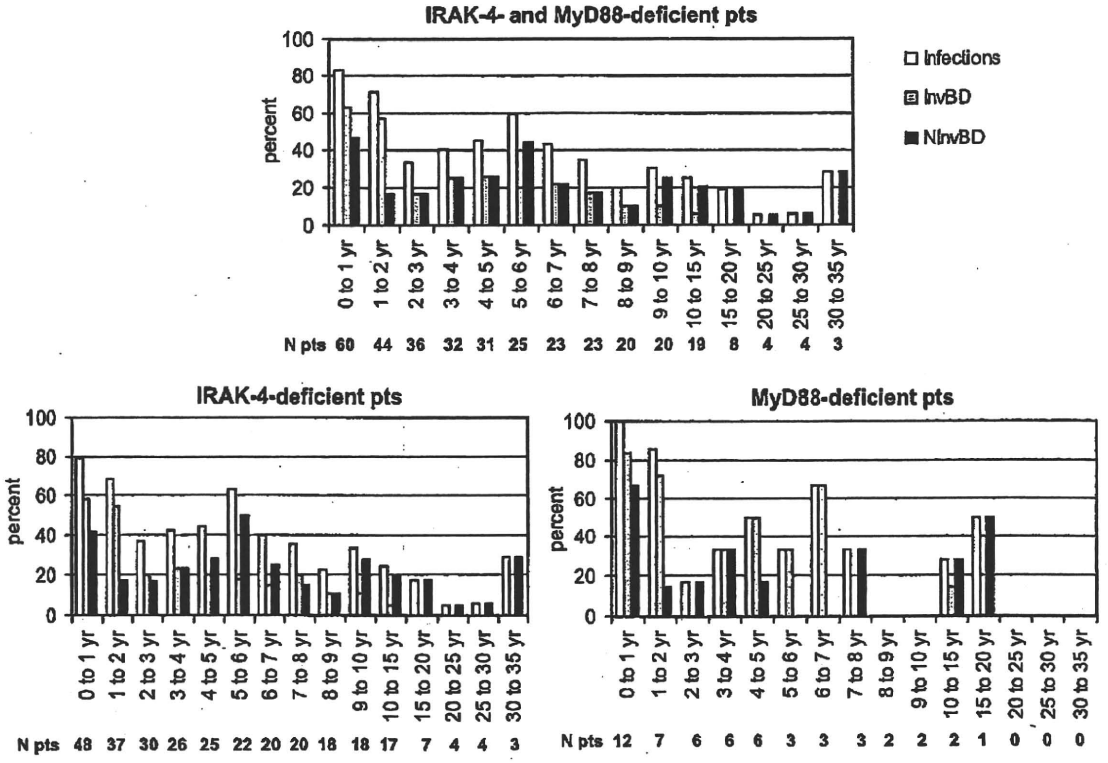

FIGURE 9. Annual rate of bacterial infections per patient, as a percentage. P = patients presenting at least 1 infection over the course of a year. Percent = P over the total number of patients.

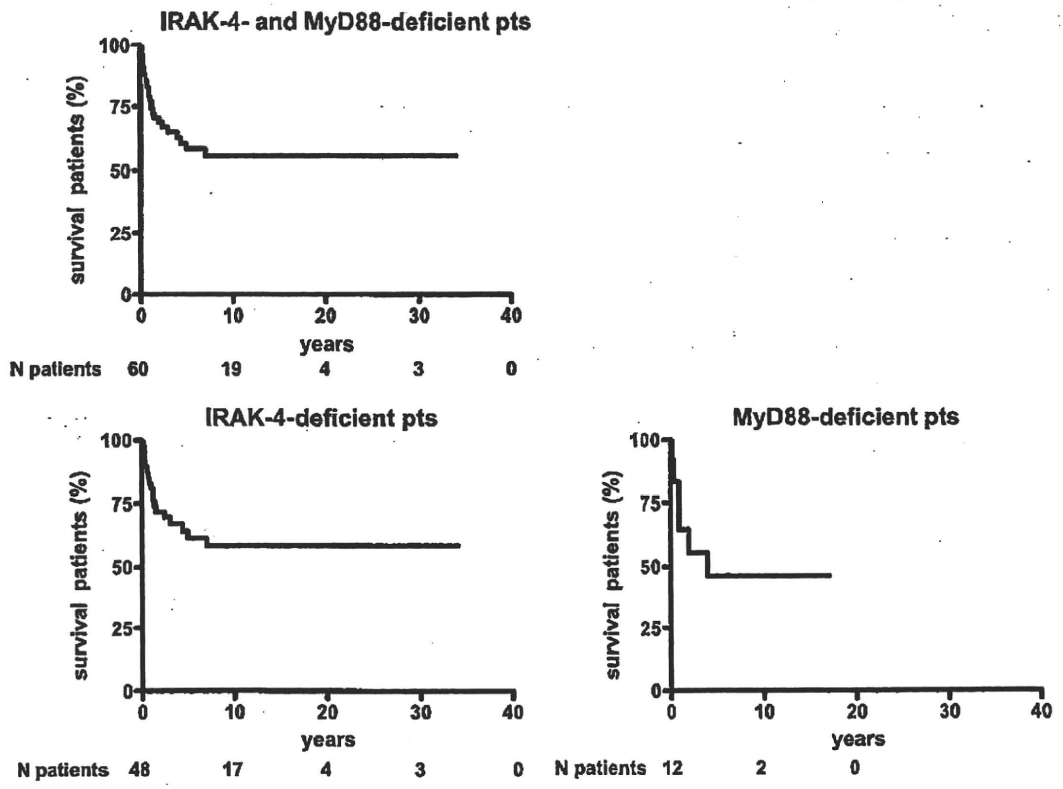

FIGURE 10. Survival curve of IRAK-4-deficient and MyD88-deficient patients.

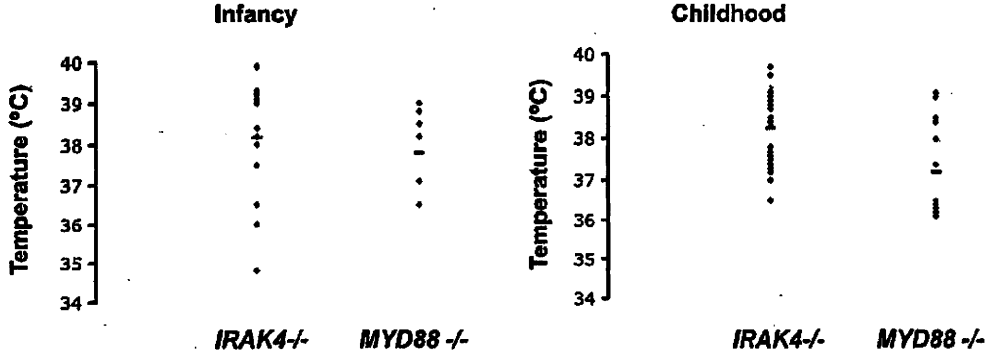

FIGURE 11. The inflammatory phenotype of IRAK-4/MyD88-deficiency. Temperature during bacterial infection in infancy and childhood.

or nonconjugated vaccine (12/48 IRAK-4-deficient patients, 7/12 MyD88-deficient patients).

We evaluated the impact of prophylaxis on the incidence of InvBD and their prognosis in all patients. Of all patients with documented bacterial infections, there was a total of 227 years and 152 years of follow-up without or with prophylaxis, respectively. At least 1 InvBD was observed in 35% of years without prophylaxis and in 16.4% of years on prophylactic treatment, and this difference was highly significant ($p = 10^{-5}$). We noted that no InvBD was documented in the 11 patients over the age of 14 years (10 IRAK-4-deficient patients and 1 MyD88-deficient patient), although only 4 of these patients continued to receive prophylactic treatment (antibiotics in 3 cases and antibiotics plus IgG infusions in the fourth case) (Figure 8; Table 10). For the 7 patients aged >14 years without prophylactic treatment, there was a total cumulative follow-up time of 49 years without any InvBD.

In conclusion, both IRAK-4 deficiency and MyD88 deficiency confer a predisposition to InvBD, mostly caused by *Str. pneumoniae*, *Staph. aureus*, and *P. aeruginosa*. In addition, both conditions confer a predisposition to NInvBD, often severe skin infections, mostly caused by *Staph. aureus*, and severe forms of ear, nose, and throat infections caused by *P. aeruginosa*. Clinical status and outcome both improve with age. There seems to be a beneficial role of prophylaxis combining intensive vaccinations, oral antibiotics, and IgG injections.

The most important advice for the families and physicians of IRAK-4-deficient and MyD88-deficient patients is to initiate empiric parenteral antibiotic treatment as soon as infection is suspected or the patient develops a moderate fever, without taking inflammatory parameters into account, because patients may die from rapid invasive bacterial infection even if prophylactic measures are taken.

DISCUSSION

We provide here the first detailed description, to our knowledge, of the clinical features and outcome of a large series of patients with IRAK-4 and MyD88 deficiencies, a novel group of primary immunodeficiencies characterized by a selective and profound defect of TLR and IL-1R signaling. Patients with these 2 deficiencies are highly susceptible to InvBD caused by *Str. pneumoniae* and *Staph. aureus*, and to NInvBD caused by *Staph. aureus* and *P. aeruginosa*. NInvBD is largely restricted to the skin (*Staph. aureus*) and the upper respiratory tract (*P. aeruginosa*). By contrast, several sites are affected during InvBD, with abscesses of inner organs, lymph nodes and saliva glands, meningitis, and septicemia frequently observed. Recurrent invasive pneumococcal disease is a hallmark of these 2 primary immunodeficiencies. Infections typically run an acute, as opposed to chronic course. However, they may be difficult to diagnose, due to weak inflammatory signs that appear late. No chronic pulmonary disease is observed in these patients, and both acute bronchitis and pneumonitis are rare. Gastrointestinal and urogenital infections are also rare.

Finally, the lack of viral, parasitic, and fungal disease in these patients is striking and cannot merely result from medical prophylaxis, as proposed elsewhere,[33] because the prophylaxis used targets mostly pyogenic bacteria, and patients with no prophylaxis do not present such infections. The nature and sites of infections in patients with IRAK-4 and MyD88 deficiencies seem to be well delineated: mostly invasive pneumococcal

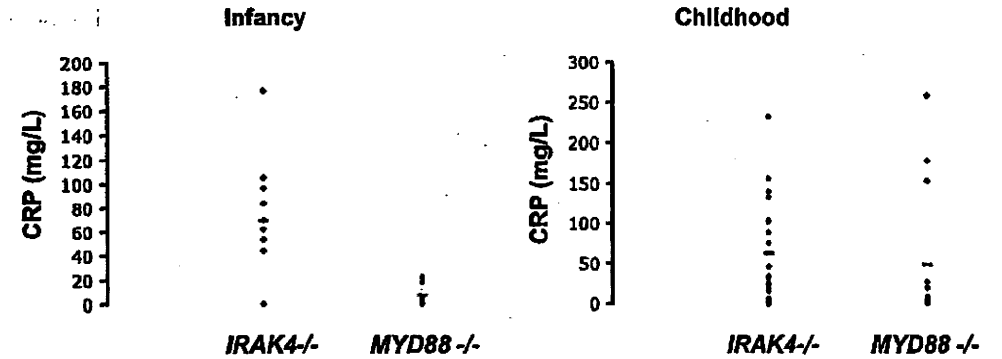

FIGURE 12. CRP concentration during bacterial infection in infancy and childhood.

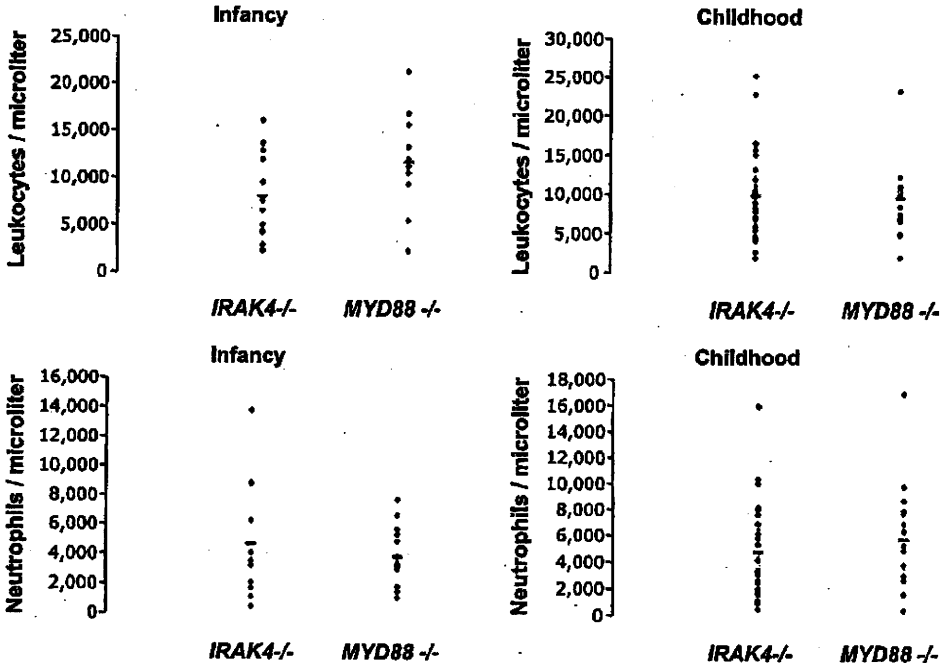

FIGURE 13. Polymorphonuclear neutrophil counts during bacterial infection in infancy and childhood.

disease, cutaneous and invasive staphylococcal disease, and *Pseudomonas* infection of the upper respiratory tract or peritoneum. It is striking that the range of infectious agents is much narrower than predicted from the mouse model of experimental infection: MyD88-deficient and IRAK-4-deficient mice are susceptible to more than 40 infectious agents.[25,45] The sites of infection also provide us with unique information about the anatomical role of the TIR pathway in host defense.

The infectious phenotype of MyD88- or IRAK-4-deficient patients is related to but different from that observed in most patients with NEMO or IκBα deficiency, who generally display impairment of both TIR-signaling and other NF-κB-dependent immunologic pathways.[7] Indeed, up to 85 patients with hypomorphic mutations of NEMO and 5 patients with hypermorphic mutations of IKBA have been reported.[7,13,21,28,31] Some of these patients had developmental signs ranging from ectodermal dysplasia with osteopetrosis and lymphoedema to a complete absence of a developmental phenotype, whereas IRAK-4-deficient and MyD88-deficient patients have no signs of developmental impairment.[7] The spectrum of infectious diseases is broad in NEMO-deficient and IκBα-deficient patients, as most patients present multiple infections, although some display a specific predisposition to pneumococcal or mycobacterial diseases.[7] Almost all patients present infections caused by pyogenic bacteria, and only a few patients suffer from mycobacterial, fungal, and/or viral diseases. The most frequent pathogens observed include gram-positive (*Str. pneumoniae* and *Staph. aureus*) and gram-negative pyogenic bacteria (*P. aeruginosa* and *H. influenzae*). Patients bearing mutations in NEMO almost invariably have an impaired antibody response to glycans, including pneumococcal capsules in particular, as in half the IRAK-4- and MyD88-deficient patients explored for antibody responses to a subset of glycan antigens.[40] Thus, the bacterial diseases seen in NEMO-deficient patients are probably due in part to the impact of NEMO mutations on the TIR-signaling pathway. Conversely, the other infections seen in NEMO-deficient patients but not in IRAK-4-deficient and MyD88-deficient patients probably reflect the impairment of other signaling pathways.

The association of clinical disease caused by *Str. pneumoniae*, *Staph. aureus*, and *P. aeruginosa* is unique among primary immunodeficiencies other than IRAK-4, MyD88, NEMO, and IκBα deficiencies.[37] Primary immunodeficiencies affecting bacterial opsonization and splenic phagocytosis are associated with invasive pneumococcal disease. These conditions include most B- and T-cell defects, congenital asplenia, deficiencies of C3, the early component of the classical and alternative complement pathway.[39] These patients develop recurrent invasive pneumococcal disease due to *Str. pneumoniae*, but are less susceptible to *Staph. aureus* and *P. aeruginosa* infections.

Other primary immunodeficiencies, such as STAT3 and TYK2 deficiencies in HyperIgE syndromes, are associated with staphylococcal infections,[7] but patients with these primary immunodeficiencies do not suffer from invasive pneumococcal disease and *Pseudomonas* infection. Notably, two-thirds of the explored IRAK-4- and MyD88-deficient patients were found to have high levels of IgE, but these levels were modest with respect to the very high IgE levels described in STAT-3-deficient patients.

Finally, most primary immunodeficiencies involving phagocyte defects, including congenital neutropenia, leukocyte adhesion deficiency, and chronic granulomatous disease, are associated with severe infections caused by *P. aeruginosa* and *Staph. aureus*, but patients with these disorders are not particularly prone to invasive pneumococcal disease.[39] A diagnosis of IRAK-4 or MyD88 deficiency or of NEMO/IκBα-related defects should be considered even with only 1 or 2 of these 3 infections. Neonates, infants, and children with invasive pneumococcal disease, severe staphylococcal disease, or *Pseudomonas* lesions of the upper respiratory tract or peritoneum, particularly in cases of recurrence, should be tested for the NF-κB pathways, including the TIR pathway in particular.[2,9] This list is not exclusive, as systemic shigellosis was

TABLE 8. Inflammatory Signs at Admission in Patients With IRAK-4 Deficiency Who Had InvBD

Age Group	Age at Onset	No. of Episodes*	Temperature (°C)			CRP (mg/L)			Whole Leukocyte Count (WLC/μL)			Neutrophil Count (NC/μL)		
			Mean	Max	SD	Mean	Max	SD	Mean	Max	SD	Mean	Max	SD
Neonatal period	7 d to 17 d	5 (T) 5 (CRP) 5 (WLC) 3 (NC)	37.2	38.0	0.6	43.6	150	61.2	9550 (N: 2700–13,000)	18,000	6807	3525	5308	11,500
Infancy	5 wk to 11 mo	16 (T) 12 (CRP) 13 (WLC) 12 (NC)	38.2	39.9	1.4	69	176.4	51.1	8034 (N: 4300–9700)	16,000	4422	4643	13,760	3999
Childhood	1 yr to 14 yr	27 (T) 22 (CRP) 36 (WLC) 31 (NC)	38.3	41.0	1.0	61.5	156	65.3	9875 (N: 4300–9700)	25,200	4894	4731	15,940	3593

*No. of patients for whom the following data were available: T = temperature, CRP = C-reactive protein concentration, WLC = whole leukocyte count, NC = neutrophil count.

TABLE 9. Inflammatory Signs at Admission in Patients With Myd88 Deficiency Who Had InvBD

Age Group	Age at Onset	No. of Episodes*	Temperature (°C)			CRP (mg/L)			Whole Leukocyte Count (WLC/μL)			Neutrophil Count (NC/μL)		
			Mean	Max	SD	Mean	Max	SD	Mean	Max	SD	Mean	Max	SD
Infancy	5 wk to 11 mo	7 (T) 12 (CRP) 14 (WLC) 12 (NC)	38.1	39.0	1.3	7.2	6.5	21.8	11,691 (N: 4300–9700)	21,300	4964	3783	7680	1998
Childhood	1 yr to 10 yr	17 (T) 14 (CRP) 16 (WLC) 15 (NC)	37.2	39.1	1.1	47.7	153	83.7	9515 (N: 4300–9700)	23,200	4694	5693	16,900	4070

*No. of patients for whom the following data were available: T = temperature, CRP = C-reactive protein concentration, WLC = whole leukocyte count, NC = neutrophil count.

TABLE 10. Prophylaxis

	IRAK-4-Deficient Patients	MyD88-Deficient Patients
Antibiotic prophylaxis	28/48	6/12
Penicillins	6	1
Cotrimoxazole	10	1
Penicillins plus cotrimoxazole	8	4
Cephalosporin	1	—
Azythromycin	1	—
Quinolone	2	—
IgG treatment	15/48	4/12
Antibiotic prophylaxis plus IgG treatment	13/48	4/12
No prophylaxis	18/48	6/12

documented in 2 patients, and other infectious diseases associated with these primary immunodeficiencies may be revealed by the investigation of other patients in the future.

In IRAK-4- and MyD88-deficient patients, clinical and laboratory signs of inflammation develop slowly even in cases of severe infection. The current study confirms and expands previous work indicating that CRP concentration, total leukocyte counts, and neutrophil numbers are typically low, but may also rise to appropriately high levels during prolonged infections, whereas temperature frequently remains inappropriately low even in such infections.[18] Thus, weak signs of inflammation despite severe infection provide a further clue to possible defects in TIR signaling, although appropriately high levels of inflammatory signs do not rule out the diagnosis of TIR deficiency.[18] Impairment of the production of IL-6-inducible molecules, such as CRP, may be observed. IRAK-4- and MyD88-deficient cells produce small amounts of IL-6 and IL-8 in vitro upon activation with IL-1 and TLR agonists.[25,38,49] As CRP contributes to the clearance of pyogenic bacteria including pneumococcus,[35,47] susceptibility to *Str. pneumoniae*, *Staph. aureus*, or *P. aeruginosa* may be increased by the slow rise in CRP levels. Similar delays in the development of signs of inflammation are observed in patients with NEMO and IκBα deficiencies, whose broader susceptibility to infections includes these pyogenic bacteria.[7]

Some IRAK-4-deficient patients (n = 10) had a delay in umbilical cord detachment and/or omphalitis. Other primary immunodeficiencies, such as leukocyte adhesion deficiency type 1 and Rac2 deficiency, have been associated with late loss of the umbilical cord and/or omphalitis, but extremely high levels of circulating neutrophils and a lack of pus formation in peripheral tissues are classically found in these disorders.[36] By contrast, in IRAK-4- and MyD88-deficient patients, impaired polymorphonuclear neutrophil mobilization and/or frank neutropenia occurs from the onset of infection, perhaps secondary to the lack of IL-8 production. Despite this neutropenia, pus formation is normal in IRAK-4- and MyD88-deficient patients. The precise mechanism of cord separation is unknown, but it does require MyD88- and IRAK-4-dependent signals, as well as CD18-expressing leukocytes. Conversely, unlike patients with various phagocyte defects, such as chronic granulomatous disease, none of the IRAK-4- and MyD88-deficient patients had inflammatory bowel disease.[36]

Despite conferring selective susceptibility to only a few bacteria, IRAK-4 and MyD88 deficiencies are nonetheless life-threatening in infancy and childhood, with a mortality rate of 38% in our series. Strikingly, however, although IRAK-4 and MyD88 appear to be vital in childhood, infections in patients lacking these proteins become rarer with age, with no death recorded in patients after the age of 8 years and no invasive infection after the age of 14 years, even in the absence of antibiotics or/and IgG prophylaxis in 7 patients over the age of 14 years. In total, this represents a cumulative time of 49 years without any InvBD for these patients. This dramatic improvement with age may be accounted for by adaptive antigen-specific T- and B-lymphocyte responses. Indeed, our patients displayed no detectable defect of protein antigen-specific T- and B-cell responses, although some patients were found to have weak antibody responses to a subset of glycan antigens.

Recent studies of neonatal bacterial sepsis in newborn mice suggest a reliance on innate immunity early in life, which progressively diminishes with age.[51] An alternative complementary hypothesis is that innate immune responses may also mature with age.[4,25] Other sensors, such as RIG-I-like helicases and NOD-like receptors, may progressively play a compensatory role. In any event, clinical improvement did not result solely from prophylaxis following diagnosis of the first infection or of the underlying deficit. The TIR pathway, including TLR responses in particular, remains dependent on IRAK-4/MyD88 with age, but the maturation of other pathways may gradually compensate for the lack of TIR signaling.

In this study, we show that the prognosis of IRAK-4 and MyD88 deficiencies is severe in infancy and early childhood, but improves substantially in adolescence. This finding is probably unique so far in the field of primary immunodeficiencies, which classically do not improve with age. This improvement with age is a hallmark of these conditions, not observed in other primary immunodeficiencies. A similar but less striking spontaneous improvement has been reported only in children with IL-12p40 and IL-12Rβ1 deficiencies.[10]

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Early and Rapid Detection of X-Linked Lymphoproliferative Syndrome with *SH2D1A* Mutations by Flow Cytometry

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Background: X-linked lymphoproliferative syndrome (XLP) is a rare immunodeficiency with extreme vulnerability to Epstein-Barr virus (EBV) infection. It presents with fatal infectious mononucleosis, lymphoproliferative disorder, or dysgammaglobulinemia. The majority of affected males have mutations in the *SH2D1A/SLAM-associated protein* (SAP) gene. We previously generated an antihuman SAP monoclonal antibody (KST-3) for a flow cytometric assay and described the activation of T cells to be necessary for the flow cytometric assessment of the SAP expression using an FITC-conjugated secondary antibody.

Methods: Between 2005 and 2008, we recruited 23 male patients with suspected XLP, including mainly EBV-associated hemophagocytic lymphohistiocytosis (HLH), and attempted to evaluate SAP expression in fresh lymphoid cells using Alexa Fluor 488-conjugated secondary antibody instead of an FITC-conjugated one.

Results: The method demonstrated that SAP was intensely expressed in $CD8^+$ T cells and NK cells in normal fresh blood samples, thus suggesting the possible rapid identification of individuals with SAP deficiency. *SH2D1A* mutations were identified in six patients with SAP deficiency, but not in patients with normal SAP expression.

Conclusion: The outcomes from this trial were verified by a flow cytometric assay using KST-3 and Alexa Fluor 488 secondary antibody. Based on the demonstration SAP deficiency in patients with suspected XLP, including mainly EBV-associated HLH, this approach could serve as a method for the early and rapid detection of patients with XLP-1. © 2010 International Clinical Cytometry Society

Key terms: flow cytometry; hemophagocytic lymphohistiocytosis; SLAM-associated protein; *SH2D1A*; X-linked lymphoproliferative syndrome; genetic analysis

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X-linked lymphoproliferative syndrome (XLP) is a rare inherited immunodeficiency estimated to affect approximately one in one million males, though it may be under-diagnosed (1). Most XLP patients die in childhood; the survival rate is very poor, even with treatment (2). Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for XLP. Rapid definitive diagnosis and appropriate treatment are extremely significant for life-saving and improved prognosis for XLP patients (3). The responsible gene is termed the *SH2D1A/SLAM-associated protein* (SAP) gene (4-6). In contrast, some presumed XLP patients do not harbor *SH2D1A* mutations, although they are clinically and even historically similar to XLP patients with *SH2D1A* mutations. Recently, Rigaud et al. (7) identified the second causative gene for XLP, the *BIRC4* gene, which encodes the X-linked inhibitor of apoptosis protein (XIAP). Therefore, XLP is now divided into two distinct diseases, XLP-1 and XLP-2.

Regarding a rapid diagnosis of XLP-1, we previously generated a rat monoclonal antibody (mAb) specific for human SAP protein, termed KST-3, to develop a flow cytometric analysis of SAP deficiency seen in XLP patients with *SH2D1A* mutations (8). In the present study, we attempt to evaluate possible SAP expression in fresh lymphoid cells with a flow cytometric assay employing Alexa Fluor 488-labled secondary antibody, which is much brighter than conventional FITC antibodies (9). Between 2005 and 2008, we used a flow cytometric determination of SAP deficiency in $CD8^+$ T and NK cells to test 23 male patients with suspected XLP, including mainly EBV-associated hemophagocytic lymphohistiocytosis (HLH). *SH2D1A* mutations were identified in six patients with SAP deficiency, but not in the other patients with normal SAP expression. These results demonstrate that a flow cytometric assay using KST-3 and Alexa Fluor 488 secondary antibody can achieve the early and rapid detection of patients with XLP-1.

MATERIALS AND METHODS

Study Subjects

The subjects in this study were largely male patients with EBV-associated HLH. In addition, a few male patients with lymphoma or hypo-γglobulinemia of unknown genetic origin were studied. A total of 23 Japanese male patients between 4 months and 40 years of age with suspected XLP-1 were tested between 2005 and 2008. Normal donors included healthy adult volunteers 24-42 years of age, and children 1-14 years of age without immunologic and hematologic diseases. After written informed consent was obtained, 5-10 mL of venous blood was collected into heparin-containing syringes and subjected to investigation within 24 h. The study was approved by the Ethics Committee of the University of Toyama.

Flow Cytometric Analysis of SAP Expression

We performed a flow cytometric analysis of SAP expressed in lymphoid cells using a rat antihuman SAP mAb, termed KST-3, as previously described (8). We employed the Alexa Fluor 488-conjugated secondary antibody to examine the possible flow cytometric assessment of SAP expression in fresh lymphoid cells. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation and immediately fixed in 1% paraformaldehyde for 30 min at room temperature, and then permeabilized in 0.5% saponin for 15 min on ice. These cells were incubated with 2 μg/ml of KST-3 (rat IgG1) or irreverent rat IgG1 for 20 min on ice and further stained with a 1:1,000 dilution of Alexa Fluor 488-conjugated goat anti-rat antibody (Molecular Probes, Eugene, OR) for 20 min on ice. To evaluate SAP expression in $CD8^+$ T cells, $CD4^+$ T cells, NK cells, and B cells, PBMC were stained with phycoerythrin-conjugated anti-CD8, anti-CD4, anti-CD56 or anti-CD19 mAbs (DAKO Japan, Kyoto, Japan), respectively, before cellular fixation and permeabilization. In some experiments, we used phycoerythrin-Texas Red (ECD)-conjugated anti-CD45RO (Immunotech, Marseille, France). We analyzed the stained cells with a flow cytometer (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan).

SH2D1A Mutation Detection

The *SH2D1A* mutations were detected by the direct sequencing. Genomic DNA was purified from PBMC with a QIAamp Blood Kit (Qiagen, Hilden, Germany), and each of the four exon-intron boundaries of the *SH2D1A* gene was amplified by PCR using the following primers: exon 1, forward, 5′-GCC CTA CGT AGT GGG TCC ACA TAC CAA CAG-3′, and reverse 5′-GCA GGA GGC CCA GGG AAT GAA ATC CCC AGC-3′; exon 2, forward, 5′-GGA AAC TGT GGT TGG GCA GAT ACA ATA TGG-3′, and reverse, 5′-GGC TAA ACA GGA CTG GGA CCA AAA TTC TC-3′; exon 3, forward, 5′-GCTCCTCTTGCAGGGAAATTC AGC CAACC-3′, and reverse, 5′-GCT ACC TCT CAT TTG ACT TGC TGG CTA CAT C-3′; exon 4, forward, 5′-GAC AGG GAC CTA GGC TCAGGC ATA AAC TGA C-3′, and reverse, 5′-ATG TAC AAA AGTCCATTT CAG CTT TGAC-3′ as previously described (6). We used the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with an automated ABI PRISM 310 DNA sequencer (Applied Biosystems) to carry out the sequence reaction.

RESULTS

SAP Expression in Normal Donors

We examined whether a flow cytometric analysis employing an Alexa Fluor 488-conjugated secondary antibody instead of an FITC-conjugated one could assess possible SAP expression in fresh lymphoid cells. We used this method to examine normal donors for SAP expression of $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and B cells in fresh blood samples. A representative profile in a healthy adult donor is shown in Figure 1. It has been shown that the SAP protein is basically expressed in all major T cell subsets and NK cells (6,8,10,11). Consistent with these observations, we demonstrated that $CD8^+$ T cells and NK cells expressed SAP intensely,

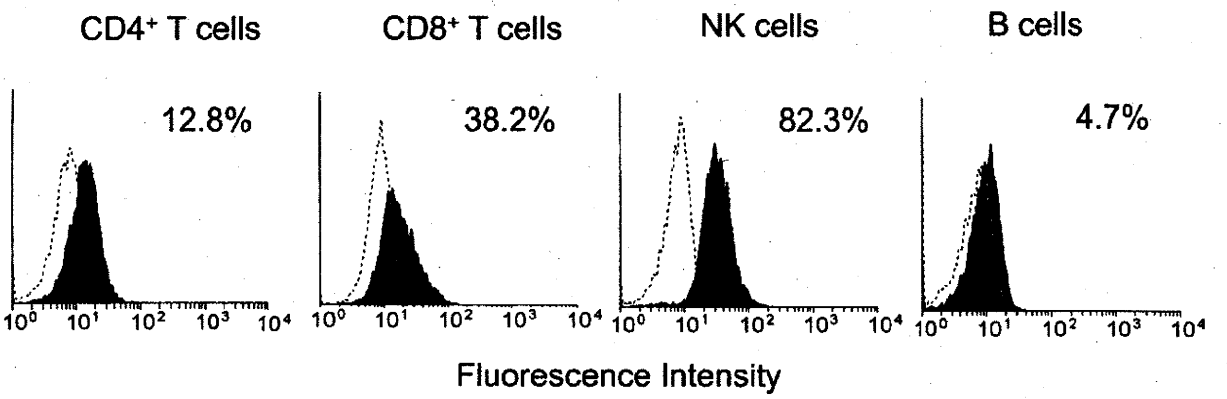

FIG. 1. The SAP expression in T, B, and NK cells in a normal adult donor. A flow cytometric analysis showed that $CD8^+$ T cells and NK cells expressed SAP relatively intensely, $CD4^-$ T cells weakly, and B cells negligibly. The dotted lines and shaded areas indicate staining by the control antibody and anti-SAP mAb (KST-3), respectively.

$CD4^-$ T cells relatively weakly, and B cells negligibly. We observed that SAP expression in $CD8^+$ T cells and $CD4^+$ T cells varied from donor to donor. We assumed that this variation might be due to individual differences in proportions of $CD45RO^+$ (memory/activated) subsets among $CD8^-$ T cells and $CD4^+$ T cells. A three-color analysis demonstrated that $CD45RO^+$ populations of T cell subsets showed enhanced SAP expression, especially of $CD8^-$ T cells (Fig. 2).

SAP Expression and *SH2D1A* Mutations in Patients with Suspected XLP

Based on the above observations in normal donors, we chose a flow cytometric analysis of SAP expression in $CD8^+$ T cells and NK cells to screen for SAP deficiency seen in XLP patients with *SH2D1A* mutations. Representative flow cytometric profiles are shown in Figure 3. All patients were simultaneously examined for a genetic analysis of the *SH2D1A* gene. The results of SAP expression and *SH2D1A* mutation analyses obtained from 23 patients with suspected XLP are summarized in Table 1. Six patients (P1–P6) demonstrated a marked reduction of SAP expression in $CD8^+$ T cells and NK cells. The percentages of SAP protein in $CD8^-$ T cells and NK cells in these patients were only 0.5–3.7% and 1.2–3.1%, respectively. *SH2D1A* mutations were confirmed in the patients with SAP deficiency. The mutations included g.23917insA, g.19528G > A (IVS2 + G>A) in sibling cases, g.357insG, deletion of exons 3–4, and g.352G > T (Ala3Ser). In contrast,

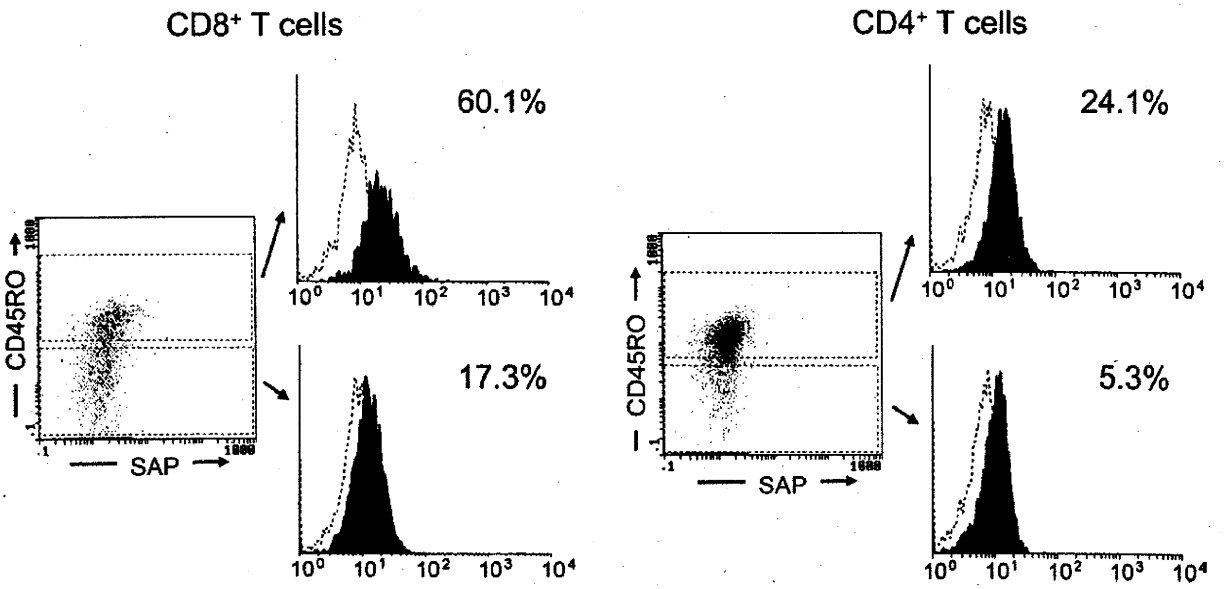

FIG. 2. An increased SAP expression in $CD45RO^+$ T cell subsets. $CD45RO^-$ (memory/activated) populations of T cells subsets, especially of $CD8^-$ T cells, exhibited an enhanced SAP expression.

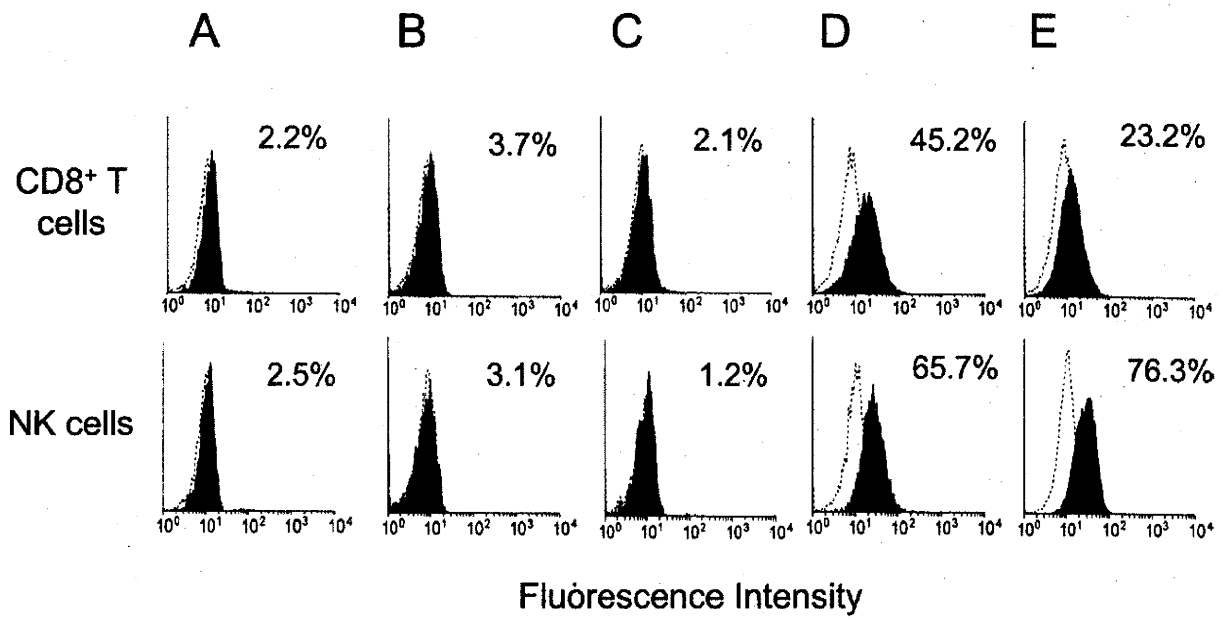

FIG. 3. The SAP expression in suspected patients with XLP. A marked SAP deficiency in both CD8+ T cells and NK cells was notable in some patients (A–C), but not in others (D, E). **A**, **B**, **C**, **D**, and **E** indicate P1, P2, P4, P10, and P11, respectively.

SH2D1A mutations were not seen in the other 17 patients (P7–P23), all of whom showed almost normal SAP expression in $CD8^+$ T cells (7.2–88.8%) and NK cells (24.3–98.1%). It is important to take into account the possibility that the flow cytometric assessment of SAP expression in $CD8^+$ T cells may be age-dependent,

Table 1
Clinical Characteristics and Immunological Data of the Patients Examined in this Study

					%SAP^- cells in		*SH2D1A* mutation	
Patient	Age	Clinical presentation	EBV	Prognosis	$CD8^+$T cells	NK cells	Nucleotide	Amino acid
P1	10 y	hypo-γ, HLH	–	After BMT	2.2	2.5	g.23917insA	Frameshift
P2	2 y	HLH	+	Dead	3.7	3.1	g.19528G > A	Frameshift
P3	2 y	ADEM	HHV-6	After BMT	0.5	1.2	g.19528G > A	Frameshift
P4	6 y	hypo-γ	–	After BMT	2.1	1.2	g.357insG	Frameshift
P5	14 y	hypo-γ, HLH, lymphoma	+	After BMT	2.2	2.7	Deletion of exons 3-4	
P6	40 y	HLH	+	Dead	2.7	NE	g.352G > T	Ala3Ser
P7	1 y	HLH	+	Alive	7.2	54.1	None	
P8	19 y	hypo-γ, gastritis	+	Alive	35.8	65.2	None	
P9	1 y	HLH	+	Alive	88.8	85.2	None	
P10	2 y	HLH	+	Alive	45.2	65.7	None	
P11	8 y	HLH	+	After CBT	23.2	76.3	None	
P12	10 mo	HLH	+	Alive	48.6	68.5	None	
P13	3 y	Lymphoma, HLH	–	Dead	70.4	98.1	None	
P14	6 y	HLH	+	Alive	35.4	55.3	None	
P15	4 mo	HLH	+	Alive	20.4	32.0	None	
P16	1 y	HLH	–	Alive	41.7	57.7	None	
P17	1 y	HLH	+	Alive	27.7	36.5	None	
P18	1 y	HLH	+	Alive	13.5	32.6	None	
P19	5 y	HLH	+	Alive	64.1	48.1	None	
P20	7 y	HLH	+	Alive	51.0	49.9	None	
P21	1 y	HLH	+	Alive	16.0	28.7	None	
P22	1 y	HLH	–	Alive	47.4	54.0	None	
P23	1 y	HLH	+	Alive	30.2	24.3	None	
Normal (n = 12)				Mean (range)	48.5 (21.6–90.8)	53.8 (23.1–94.5)		

P2 and P3 are monozygotic twins. y, years; mo, months; hypo-γ, hypogammaglobulinemia; HLH, hemophagocytic lymphohistiocytosis; ADEM, acute disseminated encephalomyelitis; HHV-6, human herpesvirus-6; BMT, bone marrow transplantation; CBT, cord blood transplantation; and NE, not examined.

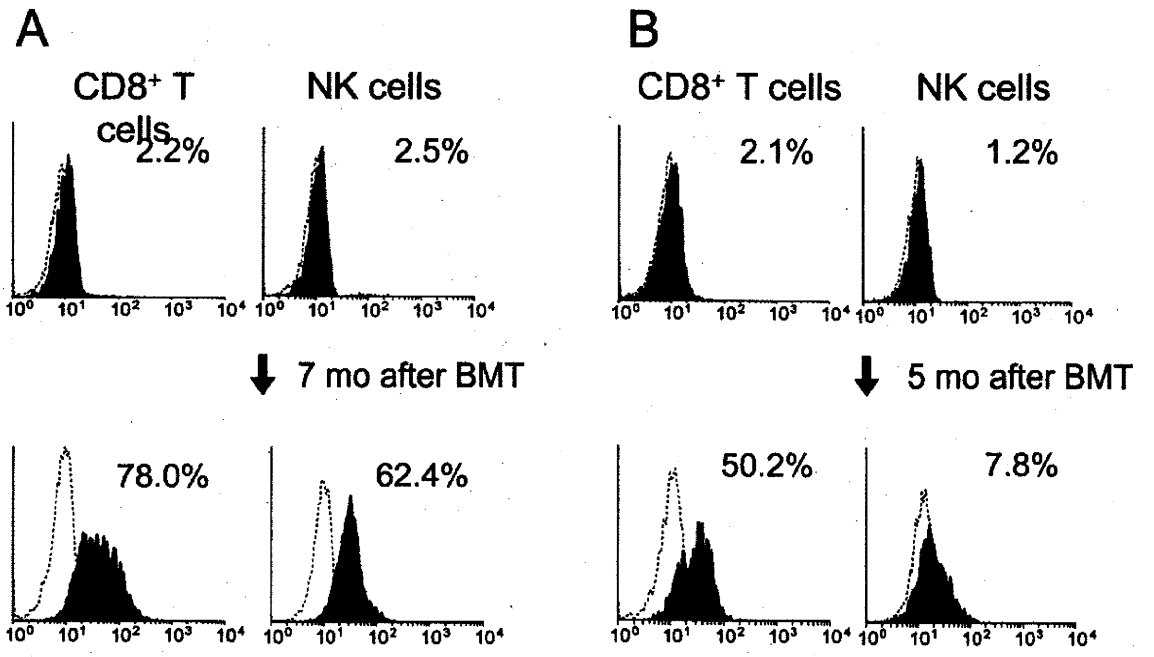

FIG. 4. The SAP expression in XLP patients after HSCT. A flow cytometric analysis demonstrated an increased SAP expression in CD8+ T cells and NK cells in 2 XLP patients after they have undergone HSCT. A and B indicate P1 and P4, respectively.

as exemplified in a one-year-old patient (P7) with no *SH2D1A* mutation. In this patient, the SAP expression in $CD8^+$ T cells was much weaker than in normal donors, thus suggesting a SAP deficiency, but the SAP expression in NK cells was comparable with the expression observed in normal donors.

Monitoring of the SAP Expression in XLP Patients After HSCT

In this series, four patients (P1, P3, P4, and P5) with XLP underwent HSCT. A flow cytometric assay was conducted to evaluate SAP expression in $CD8^+$ T cells and NK cells after HSCT. Representative cases are shown in Figure 4. All of the patients demonstrated increases of SAP expression in $CD8^-$ T cells and NK cells after undergoing HSCT. These outcomes therefore appear to validate the success of HSCT.

DISCUSSION

XLP is a rare but life-threatening disease. Most patients with XLP die by 40 years of age, and more than 70% of them die before 10 years of age (2). Early recognition in nonfamilial cases may be difficult because XLP phenotypes are heterogeneous in their clinical presentation. The ability to rapidly screen and accurately diagnose XLP patients facilitates the initiation of life-saving treatment and preparation for HSCT. Currently, XLP is divided into two distinct diseases, XLP-1 and XLP-2. The former is caused by mutations in the *SH2D1A* gene, whereas the latter is caused by mutations in the *BIRC4* gene. The majority of XLP patients have XLP-1 (7). In a previous study, we generated a rat mAb (KST-3) against human SAP protein. It was applied to the flow cytometric evaluation of SAP deficiency seen in XLP-1 patients (8). We found that activation of T cells in vitro for approximately 4 days was necessary for flow cytometric assessment of SAP expression using FITC-conjugated secondary antibody. The present study demonstrated that a flow cytometric analysis of lymphoid SAP expression was feasible in fresh blood samples by employing the Alexa Fluor 488-conjugated secondary antibody instead of the FITC-conjugated one. The Alexa Fluor 488-conjugated secondary antibody provides more intense fluorescence than the conventional one, and it can clearly discriminate positive cells from negative ones (9). Therefore, this method might lead to early and rapid detection of XLP patients with the *SH2D1A* gene.

Our flow cytometric analysis of SAP expression in $CD8^+$ T and NK cells identified SAP deficiency in 6 out of 23 patients with suspected XLP. As expected, all six patients with SAP deficiency (P1–P6) were shown to have mutations in the *SH2D1A* gene. As shown in previous studies of flow cytometry (8,11), all the missense, nonsense, and frameshift mutations in the *SH2D1A* gene resulted in deficient expression of SAP protein. Although XLP-1 patients with some missense mutations may show normal SAP expression, SAP deficiency can be demonstrated in most XLP-1 patients by flow cytometry. No *SH2D1A* mutations were identified in the remaining 17 patients with normal SAP expression. The suspected

XLP patients with normal SAP expression might have XLP-2, however, no *BIRC4* mutations were identified in these patients.

Among six patients diagnosed as having XLP-1, three patients (P2, P5, and P6) showed EBV-associated HLH, but HLH in P1 was not associated with EBV infection. Two patients (P1 and P4) showed hypo-γglobulinemia followed by acute EBV infection. P5 had EBV-negative malignant lymphoma in his brain. Interestingly, one patient (P3, a sibling of P2) had human herpesvirus-6 (HHV-6)-induced acute disseminated encephalomyelitis (ADEM). XLP is generally considered susceptible to EBV infection, but it might be vulnerable to infections from other herpesviruses as well. ADEM, is a rare manifestation in XLP, that might be a variant form of cerebellar vasculitis. Regarding clinical outcomes, two patients (P2 and P6) died of EBV-associated HLH, but four patients (P1, P3, P4, and P5) recovered after undergoing HSCT.

In conclusion, this study verified the clinical utility of a flow cytometric evaluation of lymphoid SAP expression for the detection of patients with XLP-1. Compared with the conventional Western blot technique, a flow cytometric assay can be more quickly performed with less blood, and multi-color analysis can reveal the protein expression in each cell lineage. It might be useful for detecting revertants and somatic mutations. In fact, Tabata et al. (11) demonstrated a mosaic expression of SAP in $CD8^-$ T cells, thus suggesting that the XLP-1 patient might have a revertant of $CD8^+$ T cells. Flow cytometric analysis of SAP protein is also useful to monitor a cellular reconstitution after HSCT in XLP-1 patients. Recently, a rapid flow cytometric screening method for XLP-2 has also reported (12). A male with any of the clinical phenotypes of XLP, with or without EBV infection, should initially be examined with a flow cytometric assay using both anti-SAP and anti-XIAP mAbs. Needless to say, a mutation analysis is the gold standard for confirming a definite diagnosis.

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Analysis of mutations and recombination activity in RAG-deficient patients

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Abstract Mutations in the recombination activating genes (*RAG1* or *RAG2*) can lead to a variety of immunodeficiencies. Herein, we report 5 cases of RAG deficiency from 5 families: 3 of Omenn syndrome, 1 of severe combined immunodeficiency, and 1 of combined immunodeficiency with oligoclonal TCR$\gamma\delta^+$ T cells, autoimmunity and cytomegalovirus infection. The genetic defects were heterogeneous and included 6 novel *RAG* mutations. All missense mutations except for Met443Ile in *RAG2* were located in active core regions of *RAG1* or *RAG2*. V(D)J recombination activity of each mutant was variable, ranging from half of the wild type activity to none, however, a significant decrease in average recombination activity was demonstrated in each patient. The reduced recombination activity of Met443Ile in *RAG2* may suggest a crucial role of the non-core region of RAG2 in V(D)J recombination. These findings suggest that functional evaluation together with molecular analysis contributes to our broader understanding of RAG deficiency.

1. Introduction

V(D)J recombination mediated by the recombination activating genes (RAG) 1 and RAG2 leads to the generation of diverse antigen receptors [1]. A complete lack of RAG activity causes severe combined immunodeficiency (SCID) with the

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