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X-linked thrombocytopenia (XLT) due to *WAS* mutations: clinical characteristics, long-term outcome, and treatment options

Michael H. Albert,[1] Tanja C. Bittner,[1] Shigeaki Nonoyama,[2] Lucia Dora Notarangelo,[3] Siobhan Burns,[4] Kohsuke Imai,[2] Teresa Espanol,[5] Anders Fasth,[6] Isabelle Pellier,[7] Gabriele Strauss,[8] Tomohiro Morio,[9] Benjamin Gathmann,[10] Jeroen G. Noordzij,[11] Cristina Fillat,[12] Manfred Hoenig,[13] Michaela Nathrath,[14] Alfons Meindl,[15] Philipp Pagel,[16] Uwe Wintergerst,[17] Alain Fischer,[18] Adrian J. Thrasher,[4] *Bernd H. Belohradsky,[1] and *Hans D. Ochs[19]

[1]Dr von Haunersches Kinderspital, Ludwig-Maximilians-Universität, Munich, Germany; [2]National Defense Medical College, Tokorozawa, Japan; [3]University of Brescia, Brescia, Italy; [4]University College London Institute of Child Health, London, United Kingdom; [5]Vall d'Hebron Hospital, Barcelona, Spain; [6]The Queen Silvia Children's Hospital, Göteborg, Sweden; [7]Centre Hospitalier Universitaire Angers, Angers, France; [8]Charité Campus Virchow-Klinikum, Otto-Heubner-Zentrum für Kinder- und Jugendmedizin, Berlin, Germany; [9]Tokyo Medical and Dental University, Tokyo, Japan; [10]Universitätsklinikum Freiburg, Freiburg, Germany; [11]St Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; [12]Centre de Regulació Genòmica, Centro de Investigación Biomédica en Red de Enfermedades Raras, Barcelona, Spain; [13]Universitätsklinik für Kinder- und Jugendmedizin Ulm, Ulm, Germany; [14]University Children's Hospital, Technische Universität, Munich, Germany; [15]Frauenklinik am Klinikum rechts der Isar, Technische Universität, Munich, Germany; [16]Lehrstuhl für Genomorientierte Bioinformatik, Wissenschaftszentrum Weihenstephan, Technische Universität, Freising, Germany; [17]Krankenhaus St. Josef, Braunau, Austria; [18]Hôpital Necker Enfants Malades, Paris, France; and [19]University of Washington, Seattle Children's Hospital

A large proportion of patients with mutations in the Wiskott-Aldrich syndrome (WAS) protein gene exhibit the milder phenotype termed X-linked thrombocytopenia (XLT). Whereas stem cell transplantation at an early age is the treatment of choice for patients with WAS, therapeutic options for patients with XLT are controversial. In a retrospective multicenter study we defined the clinical phenotype of XLT and determined the probability of severe disease-related complications in patients older than 2 years with documented *WAS* gene mutations and mild-to-moderate eczema or mild, infrequent infections. Enrolled were 173 patients (median age, 11.5 years) from 12 countries spanning 2830 patient-years. Serious bleeding episodes occurred in 13.9%, life-threatening infections in 6.9%, autoimmunity in 12.1%, and malignancy in 5.2% of patients. Overall and event-free survival probabilities were not significantly influenced by the type of mutation or intravenous immunoglobulin or antibiotic prophylaxis. Splenectomy resulted in increased risk of severe infections. This analysis of the clinical outcome and molecular basis of patients with XLT shows excellent long-term survival but also a high probability of severe disease-related complications. These observations will allow better decision making when considering treatment options for individual patients with XLT. (*Blood*. 2010;115(16): 3231-3238)

Introduction

In 1937 Wiskott described a clinical entity characterized by thrombocytopenia, eczema, bloody diarrhea, and recurrent otitis media in male infants. After rediscovery in 1954 by Aldrich as an X-linked recessive disorder, it was designated the Wiskott-Aldrich syndrome (WAS).[1-3] X-linked thrombocytopenia (XLT), sometimes associated with mild eczema and/or infections, was recognized in the 1960s and was suspected to be a variant of WAS.[4-6] This was confirmed when patients with XLT were shown to have mutations in the Wiskott-Aldrich syndrome protein gene (*WAS*).[7-9]

WAS gene mutations result in 3 distinct clinical phenotypes: classic WAS, XLT, and X-linked neutropenia,[10,11] and a strong genotype phenotype correlation has been suggested.[12-15] Mutations completely averting WAS protein (WASP) expression typically lead to the classic phenotype. Missense mutations resulting in expression of defective WASP, often in reduced quantity, most often result in the XLT phenotype, sometimes with only intermittent thrombocytopenia.[16] X-linked neutropenia is caused by gain of function mutations resulting in constitutively activated WASP.[17-19] There are however exceptions to these rules, making it difficult to predict the clinical course of a male infant solely based on the type of *WAS* gene mutation and its effect on WASP expression.

The classic WAS phenotype with microthrombocytopenia, severe eczema, increased susceptibility to pyogenic and opportunistic infections, and increased risk of autoimmune disease and cancer usually leads to death in early childhood or adolescence if left untreated.[10,20,21] Curative treatment by allogeneic hematopoietic stem cell transplantation (HSCT) should be offered to all such patients. The outcome is excellent if performed early in life from a human leukocyte antigen–matched related or unrelated donor.[10,22-24] Hematopoietic stem cell gene therapy might in the future offer an alternative approach in patients lacking a suitable donor.[25-27]

Generally accepted treatment policies do not exist for patients exhibiting the XLT phenotype, in whom HSCT would seem like an excessively risky procedure if they have thrombocytopenia and

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*B.H.B. and H.D.O. contributed equally to this study.

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eczema only. Although it has been assumed that patients with XLT have a lower risk of cancer or autoimmunity than patients with WAS, this has never been formally examined. Therefore, the risk–benefit ratio for HSCT is not known in XLT.

In this multicenter study we assessed retrospectively the spectrum of clinical phenotypes, the associated genotypes, and the long-term outcome of the largest cohort of patients with XLT studied so far.

Methods

Data accrual

Questionnaires were sent worldwide to major centers treating patients with primary immunodeficiency diseases (PIDs), asking to enroll their patients with the XLT phenotype and to provide data on the following disease parameters: infections, eczema, thrombocytopenia, bleeding, malignancy, autoimmunity, *WAS* gene mutation, WASP expression, and type and extent of therapy. An alternative possibility was documentation online with the same questionnaire in the European Society for Immunodeficiencies registry (www.esid.org). Patient information was made anonymous by the submitting physician. The study was approved by the ethics committee of the University of Munich, Germany.

Patients

All submitted patient data were evaluated, and patients were included as study patients by consensual decision of a central review board (M.H.A., T.C.B., B.H.B., H.D.O.). To be enrolled into the final study, patients had to fulfill all of the following criteria: (1) confirmed mutation within the *WAS* gene; (2) classified by their treating physician as having XLT; (3) with or without mild-to-moderate eczema or mild, infrequent infections not resulting in sequelae; (4) age older than 2 years; and (5) no severe infection, autoimmunity, or malignancy within the first 2 years of life.

Bleeding events before the age of 2 years were no reason for exclusion from the study. Older than 2 years, severe infections, the development of autoimmunity, or malignancy was recorded and included in the analysis, but it was no reason for exclusion from the study.

If patients underwent allogeneic HSCT, the transplantation was recorded as the last date of follow-up; the resulting events/outcome were not part of this analysis.

Definitions

Life-threatening infections were defined as requiring hospitalization such as sepsis, meningitis, or pneumonia needing oxygen supply or mechanical ventilation. Serious bleeding was defined as a fatal or life-threatening bleeding episode resulting in hospitalization or red blood cell transfusion. Other serious complications were a diagnosis of autoimmunity, malignancy, or death. If a patient experienced more than 1 serious event, only the first event was registered for the analysis of event-free survival. Severity of thrombocytopenia was defined as follows: less than 20.0×10^9/L (20 000/μL) was severe, 20.0 to 50.0×10^9/L (20 000 to 50 000/μL) was moderate, and greater than 50.0×10^9/L (50 000/μL) or cyclic was mild. All patients with normal or reduced levels of WASP detectable by Western blot or fluorescence-activated cell sorting were designated as WASP positive; those with truncated (by Western blot) or undetectable protein were categorized as WASP negative. Intravenous immunoglobulin (IVIG) or antibiotic (AB) prophylaxes were defined as having had IVIG or prophylactic ABs more than once for any period of time.

Mutations are reported according to the current nomenclature of the Human Genome Variation Society (www.hgvs.org).[28]

Statistical analysis

Kaplan-Meier survival estimates and cumulative incidence rates were compared with the use of the log-rank test (Prism; GraphPad Software Inc). Cumulative incidence for different events adjusting for competing risks was estimated with the use of the statistics language R[29] with the cmprsk package that used the method by Gray.[30] Other analyses used the χ^2 or Fisher exact test and were accepted as significantly different at a level of P less than .05.

Results

Study cohort

A total of 69 centers known to treat patients with PID were contacted and 50 responded (72%). Of 213 completed forms, representing 12 countries from 4 continents, 173 (171 male, 2 female) patients from 128 families and 21 centers with a median age of 11.5 years (range, 2.0-74.6 years) fulfilled the inclusion criteria, covering 2830 patient-years. The 2 female patients of our XLT cohort had been reported previously, 1 with a homozygous missense mutation and 1 with a heterozygous missense mutation and skewed X-inactivation in favor of the mutated allele.[31,32]

Mutations in patients with XLT

We identified 62 unique mutations (Table 1), including 3 mutational hotspots, defined as affecting 10 or more nonrelated families with either the identical mutation or a missense mutation affecting the same amino acid. Two hotspots were located in exon 2 affecting either a valine at position 75 (p.Val75Met or p.Val75Leu; 23 patients) or an arginine at position 86 (p.Arg86Gly, p.Arg86Cys, p.Arg86His, or p.Arg86Leu; 33 patients). The third hotspot mutation, located in intron 6 (c.559 + 5G>A) was found in 15 patients. Thus 41% of all patients had a hotspot mutation.

The majority of mutations was located in exon 1 (10% of all patients) and exon 2 (54%). Most mutations were missense (69% of all patients), followed by splice site mutations (19%), deletions (5%), insertions (3%), nonsense mutations (2%), and no-stop mutations (1%; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). With few exceptions, patients with missense and splice site mutations expressed WASP in reduced quantity or in truncated form (Table 1).

Survival

Without curative treatment classic WAS results in premature death, often during childhood.[21,33] Patients with XLT are expected to have a better prognosis. To verify this perception, we defined the probability of survival in our cohort of patients with XLT.

Overall survival was excellent with 97% (95% confidence interval [95% CI], 95%-100%), 96% (95% CI, 91%-100%), 81% (95% CI, 66%-97%), and 81% (95% CI, 66%-97%) at 15, 30, 45, and 60 years, respectively, and only slightly reduced compared with the survival curve of the normal male German population[34] (Figure 1A). However, survival probability without having experienced a severe disease-related event was less favorable with 74% (95% CI, 65%-82%), 56% (95% CI, 43%-70%), 36% (95% CI, 20%-53%), and 27% (95% CI, 10%-44%) at 15, 30, 45, and 60 years, respectively (Figure 1B).

Thus the excellent survival in patients with XLT is associated with a high rate of severe disease-related events throughout life.

Incidence of severe disease-related events

To better define the nature and occurrence of severe disease-related events, we analyzed the cumulative incidence rate of these events separately.

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Table 1. *WAS* gene mutations in patients with XLT

Exon	Coding DNA mutation	Predicted protein change	Mutation type	Pt*	Fam†	Origin	WASP expression (no. of pt)	Score (no. of pt)
1	c.G5C	p.Ser2Thr	Missense	1	1	Fr	ND	2
1	c.G18A	p.Met6Ile	Missense	2	1	JPN	Reduced (2)	1, 2→5M
1	c.C71T	p.Ser24Phe	Missense	2	2	US (1), JPN (1)	Reduced (1), ND (1)	1, 2→5A
1	c.C79T	p.Leu27Phe	Missense	1	1	US	Reduced	1
1	c.88_90delCAC	p.His30del	Deletion	5	2	UK (4), Ger (1)	Reduced (3), ND (2)	1(4), 2
1	c.G91A	p.Glu31Lys	Missense	1	1	Italy	Absent	2→5A
1	c.T116C	p.Leu39Pro	Missense	6	4	US (3), Italy (2), Ger (1)	Reduced (5), absent (1)	1, 1→5A/M, 2(4)
2	c.C134T	p.Thr45Met	Missense	13	8	JPN (4), US (2), Ger (1), UK (1), Sw (5)	Reduced (6), absent (1), ND (6)	1(6), 1→5A, 2(4), 2→5A /B (2)
2	c.C140A	p.Ala47Asp	Missense	1	1	US	Reduced	2
2	c.A142G	p.Thr48Ala	Missense	1	1	JPN	Reduced	2
2	c.C143T	p.Thr48Ile	Missense	1	1	US	Reduced	1→5M
2	c.C167T	p.Ala56Val	Missense	5	4	US (3), Italy (1), JPN (1)	Reduced (4), ND (1)	1(3), 1→5A, 2
2	c.C172A	p.Pro58Thr	Missense	2	1	US	Normal (2)	1, 2
2	c.C172G	p.Asp58Ala	Missense	1	1	US	Reduced	2→5A/M
2	c.C173G	p.Pro58Arg	Missense	3	1	Italy	Reduced (2), ND (1)	1, 1→5M, 2
2	c.G199A	p.Glu67Lys	Missense	1	1	Fr	Reduced	2
2	c.G223A	p.Val75Met	Missense	22	16	Fr (6), UK (5), US (5), Ger (2), JPN (2), Sp (1), Italy (1)	Normal (1), reduced (10), absent (3), ND (8)	1(6), 1→5A, 2(14), 2→5A
2	c.G223T	p.Val75Leu	Missense	1	1	US	ND	2
2	c.A227C	p.Lys76Thr	Missense	2	2	US	Reduced (1), ND (1)	2(2)
2	c.G229C	p.Asp77His	Missense	1	1	Italy	Reduced	1
2	c.A230G	p.Asp77Gly	Missense	2	1	Italy	Reduced (2)	1, 2
2	c.A239G	p.Gln80Arg	Missense	1	1	Rus	Reduced	2
2	c.248insA	p.Tyr83X	Insertion	1	1	Fr	ND	2
2	c.C256G	p.Arg86Gly	Missense	1	1	US	Reduced	2→5A
2	c.C256T	p.Arg86Cys	Missense	24	18	US (10), Ger (6), JPN (3), UK (3), Italy (1), Sw (1)	Normal (3), reduced (9), ND (12)	1(10), 1→5M, 2(12), 2→5A
2	c.G257A	p.Arg86His	Missense	7	7	JPN (2), Fr (1), Ger (1), Isr (1), Rus (1), US (1)	Reduced (4), absent (1), ND (2)	1→5A, 2(4), 2→5A(2)
2	c.G257T	p.Arg86Leu	Missense	1	1	US	Absent	2
2	c.A263G	p.Tyr88Cys	Missense	1	1	NL	ND	2→5A
2	c.G266A	p.Gly89Asp	Missense	1	1	UK	Normal	1
3	c.A320G	p.Tyr107Cys	Missense	1	1	US	Reduced	2
3	c.326_330insC	p.Thr111HisfsX9	Insertion	1	1	US	Absent	2
3	c.G355A	p.Gly119Arg	Missense	1	1	NL	ND	1
4	c.dup355_361	p.Asp121insGD	Insertion	1	1	JPN	Absent	2
4	c.G399T	p.Glu133Asp	Missense	1	1	US	Reduced	2
5	c.G505T	p.Asn169X	Nonsense	1	1	JPN	Reduced	2→5M
6	c.G538A	p.His180Asn	Missense	1	1	Italy	Reduced	1
7	c.C707G	p.Ala236Gly	Missense	1	1	Italy	Absent	1
7	c.A724T	p.Ser242Cys	Missense	1	1	NL	ND	1
9	c.854_855insG	p.Thr286AspfsX1	Insertion	2	1	UK	Reduced and truncated (1), absent (1)	1(2)
9	c.A919G	p.Met307Val	Missense	1	1	Ger	ND	2
10	c.C961T	p.Arg321X	Nonsense	1	1	JPN	Absent	2→5M
10	c.983_984delC	Multiple products	Deletion	1	1	US	Reduced and truncated	2
10	c.991insA	p.Gly334X	Insertion	1	1	US	Absent	2
10	c.1073_1074delGA	p.Gly358AlafsX135	Deletion	1	1	US	Reduced and truncated	2
10	c.1079delC	p.Pro360HisfsX84	Deletion	2	2	Ger, JPN	Reduced (1), absent (1)	2(2)
10	c.C1090T	p.Arg363X	Nonsense	2	1	Fr	ND (2)	2(2)
11	c.G1430A	p.Arg477Lys	Missense	1	1	Sp	Reduced	2
11	c.T1442A	p.Ile481Asn	Missense	2	1	Italy	Normal (1), reduced (1)	1(2)
12	c.G1453A	p.Asp485Asn	Missense	1	1	US	Reduced	2→5A
12	c.A1454G	p.Asp485Gly	Missense	3	1	Sp	ND (3)	1(3)
12	c.G1508C	p.X503SerextX76	No-stop	2	1	US	Absent (1), ND (1)	2(2)
Int 3	c.360+1G>A	p.Ala92_Asp120del	Splice (donor site)	1	1	JPN	Reduced	2
Int 3	c.361-1G>A	p.fsX201	Splice (acceptor site)	1	1	US	Reduced	2
Int 4	c.[463+1_463+8del; 464-3_464-2insG]	p.fsX178/fsX251	Splice (donor +acceptor site)	1	1	JPN	Reduced	2
Int 6	c.559+5G>A	70% fsX190/30% normal	Splice (donor site)	15	11	US (9), Ger (2), JPN (3), UK (1)	Reduced (12), absent (1), ND (2)	1(6), 1→5M, 2(6), 2→5A(2)
Int 7	c.734+5G>A	ND	Splice (donor site)	4	1	Ger	ND (4)	2 (3), 2→5A
Int 7	c.735-25A>C	ND	Splice (acceptor site)	3	1	UK	Reduced (3)	1(3)
Int 8	c.777+1G>A	p.fsX246	Splice (donor site)	2	2	Australia, US	Absent (1), ND (1)	1, 2
Int 8	c.777+3insT	ND	Splice (donor site)	2	1	Italy	Reduced (2)	1, 2
Int 8	c.778-6G>A	ND	Splice (acceptor site)	1	1	UK	Reduced	1
Int 9	c.(931_932)ins250	ND	Splice site	1	1	JPN	Reduced	1
Int 11	c.(1484_1485)ins118	Normal and abnormal splice products	Splice site	2	1	JPN	Reduced (2)	2→5A(2)

Pt indicates number of patients with the respective mutation; Fam, number of families with the respective mutation; 1→5, WAS score progressing from 1 to 5 because of either A, autoimmunity, or M, malignancy; Fr, France; ND, not done; JPN, Japan; US, United States of America; UK, United Kingdom; Ger, Germany; Sw, Sweden; Sp, Spain; Rus, Russia; Isr, Israel; and NL, The Netherlands.

*There was a total of 173 patients.

†There was a total of 128 families.

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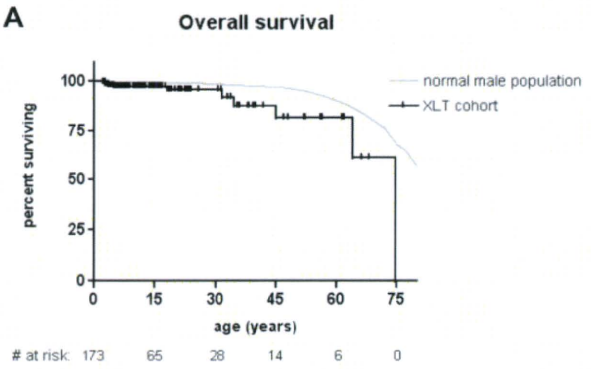

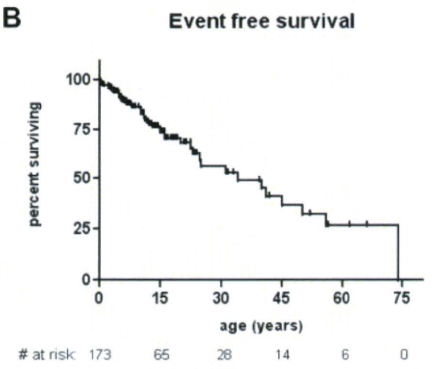

Figure 1. Overall and event-free survival. (A) Kaplan-Meier estimate of overall survival probability of all study patients compared with survival of the normal German male population 2006.[34] (B) Event-free survival probability. Event was defined as a severe or fatal infection, severe or fatal bleeding, autoimmunity, malignancy, or death. Each hash mark on a graph line indicates a censored event; # at risk, number of patients at risk at indicated time point.

Median event-free survival was 10.2 years (range, 0.1-73.9 years). A total of 86 events in 47 patients were reported, some of them occurring in different event categories in the same patient (detailed in Table 2). Cumulative incidences for each event category are detailed separately in Figure 2. If events were analyzed honoring other events as competing, the cumulative incidences were slightly lower because later events in the same patient were ignored (data not shown).

Table 2. Disease-related events

	Total events	Fatal events
Infections*		
Pneumonia	6	0
Bacterial meningitis	4	0
Sepsis	4	2
Gastrointestinal (salmonellosis)	1	1
Orchitis	1	0
Tuberculosis	1	0
No. of events	17†	3‡
No. of patients	12	3
Bleeding§		
ICH‖	18	3
Gastrointestinal	6	1
Ear/nose/throat	4	0
Pulmonary	2	1
Traumatic, not ICH	2	0
Retinal	1	0
No. of events	33	5
No. of patients	24	5
Autoimmunity¶		
Nephropathy	9	0
AIHA	6	0
Vasculitis	3	0
ITP	4	0
Arthritis	3	0
Colitis	1	0
No. of events	26	0
No. of patients	21	0
Malignancy#		
Lymphoma/EBV-LPD	4	1
MDS	1	0
Spinalioma	2	0
Seminoma	1	0
ALL	1	0
Pancreatic cancer	1	1
No. of events	10	2
No. of patients	9	2

ICH indicates intracranial hemorrhage; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenic purpura; ALL, acute lymphoblastic leukemia; EBV-LPD, Epstein-Barr virus–associated lymphoproliferative disease; and MDS, myelodysplastic syndrome.

*Three patients had more than 1 infectious event.

†Eight events were in patients who had undergone a previous splenectomy.

‡Two events were in patients who had undergone a previous splenectomy.

§Four patients had more than 1 bleeding episode.

‖Fifteen were spontaneous, 3 were traumatic.

¶Three patients had more than 1 autoimmune disease.

#One patient had 2 malignancies.

Life-threatening infections occurred at a median age of 24.8 years (range, 2.0-73.9 years), 3 of which were fatal. There was no discernible effect of patient age on the incidence of infectious events (Figure 2A). In contrast, all but 1 serious hemorrhage occurred before the age of 30 years, at a median age of 5.7 years (range, 0.1-74.6 years; Figure 2B). Most serious bleeding events (18 of 33) were intracranial hemorrhages. Five bleeding episodes were fatal at a median age of 4.9 years (range, 2.0-74.6 years). There was no correlation between the recorded platelet counts and the incidence of severe or fatal bleeding, which was 12.5% in mild, 9.7% in moderate, and 18.4% in severe thrombocytopenia ($P = .31$). Autoimmune nephropathy and hemolytic anemia were the most frequent autoimmune manifestations; the former occurring more frequently in Japanese patients than in patients from other countries (5 of 28 vs 4 of 145; $P = .006$). In general, autoimmune diseases were not significantly more frequent in Japanese patients (5 of 28 vs 16 of /145; $P = .34$). Autoimmunity was not restricted to adult patients but occurred at all ages with a median of 12.2 years (range, 4.9-56.0 years; Figure 2C). Malignancies developed at a median age of 34.0 years (range, 7.8-74.0 years; Figure 2D), half (5 of 10) of which were of lymphoid origin. Two patients died of their malignancies, 2 more went on to have HSCT and died of transplantation-related causes and 2 died of other complications.

In conclusion, with the exception of severe bleeding, which seems to be limited to the first 3 decades of life, a relatively high rate of life-threatening or fatal disease-related events was observed in XLT at all ages.

Influence of WAS gene mutation, protein expression, IVIG, or AB prophylaxis on overall and event-free survival

Because some patients with XLT have a largely uneventful course of disease and a normal life expectancy and others have severe or even fatal complications at any age, we asked whether individual *WAS* gene mutations, the presence or absence of WASP, or the prophylaxis with ABs and intravenous immunoglobulin had any influence on outcome.

WASP expression, if assessed, was detectable in 98 patients and absent in 21. Presence or absence of WASP had no influence on overall and event-free survival in patients with the XLT phenotype (Figure 3A). Similarly, there was no significant effect on the incidence of disease-related events (data not shown). The same was true when the influence of IVIG prophylaxis (n = 39) was analyzed in comparison to patients having never received IVIG (n = 134; Figure 3B). AB prophylaxis had no positive influence on

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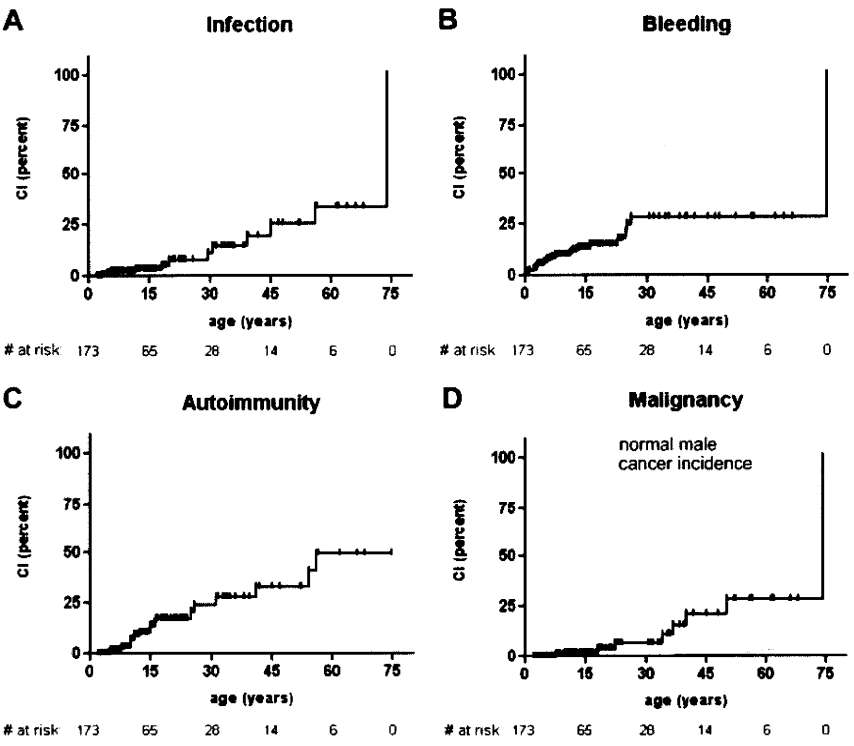

Figure 2. Cumulative incidence rate of severe events. Cumulative incidence of (A) severe or fatal infectious episodes in the study cohort, (B) severe or fatal bleeding episodes, (C) autoimmune disease, and (D) malignancy, compared with cancer incidence in the US male population.[35] Each hash mark on a graph line indicates a censored event, # at risk, number of patients at risk at indicated time point.

outcome (Figure 3C). Patients with hotspot mutations had no different overall and event-free survival and event incidences compared with others (data not shown).

In summary none of the tested outcome variables were of significance in this cohort of patients with XLT selected on the basis of their mild phenotype.

Influence of splenectomy on infections and bleeding episodes

Splenectomy in patients with XLT/WAS usually leads to a sustained increase in platelet counts and is considered an effective measure to control the bleeding predisposition. Therefore, splenectomy has been recommended by some investigators for patients with WAS and patients with XLT.[36,37]

A total of 41 patients (23.7%) underwent splenectomy at a median age of 7.02 years (range, 0.8-43.0 years). The indication for splenectomy was not reported, but 7 of these 41 patients had experienced a severe bleeding episode before splenectomy, and 28 of 41 patients had had severe thrombocytopenia. All 13 patients in whom postsplenectomy platelet counts were available had experienced an increase in platelet numbers, 7 having counts greater than 100.0×10^9/L (100 000/μL). In the 2 patients who experienced a severe bleeding event after splenectomy, platelet counts were not reported. Therefore, it cannot be excluded that these 2 patients may have had low counts despite splenectomy. The overall cumulative incidence rate of serious bleeding events in these patients after splenectomy compared with before splenectomy was reduced although not significantly ($P = .15$). However, there was a significantly higher incidence of severe infectious events after splenectomy than before ($P = .005$). This might possibly be due to negligent AB prophylaxis in some patients. Of the 9 patients who did not receive AB prophylaxis, 3 had a severe (1 fatal) infection up to 53 years after splenectomy. This compared unfavorably, however not statistically significant, to patients who underwent splenectomy with AB prophylaxis in whom only 5 of 32 (1 fatal) had such an event ($P = .34$). Overall survival in patients who underwent splenectomy was not significantly different from patients not undergoing splenectomy (data not shown).

These data indicate that patients with XLT who underwent splenectomy are at significant risk of severe infections and require life-long AB prophylaxis.

Discussion

WAS is a multifaceted disorder with a wide spectrum of disease severity. In contrast to classic WAS, patients with a mild clinical phenotype, termed XLT, require comprehensive assessment in deciding on the strategy to provide optimal treatment. This is true for children who often present with selective microthrombocytopenia and have an uncertain long-term prognosis at a time when they are excellent candidates for allogeneic HSCT.[23,24] Similarly, adult patients with XLT who often are wrongly categorized as having chronic immune thrombocytopenic purpura and who may already have developed complications such as autoimmunity pose unique therapeutic challenges. This retrospective study was designed to better define the type of mutations and the clinical course of patients with XLT and to collect supportive evidence for optimal treatment choices.

The design of such a study requires a stringent definition of inclusion and exclusion criteria. The WAS scoring system has been used successfully in categorizing patients according to their disease severity.[10,11] However, an individual patient is not expected to keep the same score throughout his or her life. Progression from a score of 1 to 4 to a score of 5 by developing cancer or autoimmunity can occur at any age, and patients with classic WAS often present with a relatively mild phenotype during infancy. We, therefore, chose inclusion criteria that best reflect the situation when patients with XLT/WAS present in an immunodeficiency clinic. In addition to the classification as XLT by physicians experienced in treating patients with PIDs, we deliberately chose stringent criteria to prevent the

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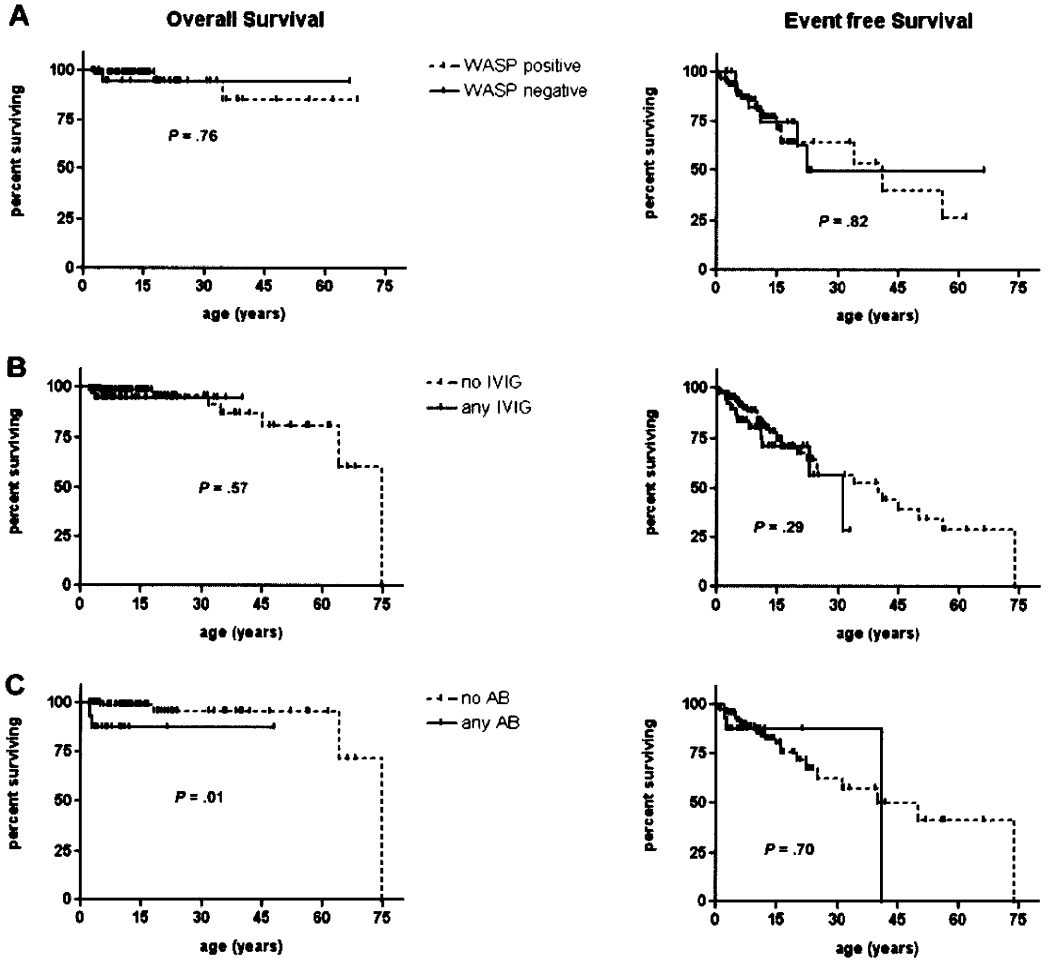

Figure 3. Influence of WASP expression, IVIG, or AB prophylaxis on overall and event-free survival. Kaplan-Meier estimate of overall survival and event-free survival probability of (A) WASP-positive (n = 98, dotted line) and WASP-negative (n = 21, solid line) patients. (B) Patients receiving any IVIG prophylaxis (n = 39, solid line) or no IVIG prophylaxis (n = 134, dotted line) and (C) patients receiving any AB prophylaxis (n = 16, solid line) or no AB prophylaxis (n = 116, dotted line). Patients who underwent splenectomy were excluded from the analysis in panel C. Each hash mark on a graph line indicates a censored event.

inclusion of patients with classic WAS with few disease symptoms as may be the case during the first 2 years of life. One possible drawback of this study could be its retrospective, cross-sectional design. It is probable that some events took place when medical care differed from that of today. Naturally, the study design might encompass a bias by some confounding factors such as patient compliance, physician preference, choice of prophylactic measures, and availability of HSCT. We can also not exclude some selection bias, missing very mild cases that are undiagnosed or misdiagnosed and not referred to an immunology center. But some older patients in this study had lived an uneventful life, before being diagnosed as XLT because their brothers, nephews, or grandsons were discovered to have a *WAS* gene mutation. Of note, the outcome of these older relatives did not differ from that of the rest of the cohort (data not shown). At this time the retrospective study design seems to be the only possible means to assess the clinical characteristics of a large cohort of patients with XLT. Having established this database of patients with XLT, we now have the opportunity to prospectively follow their course of disease.

Only 17.6% of evaluable patients with XLT from this cohort lacked WASP expression. In contrast, the proportion of WASP-negative patients from a multinational cohort of patients with WAS/XLT with known *WAS* mutations was 57% (104 of 184).[15] Some patients may in fact express WASP because the methods used to assess expression, such as Western blot analysis, might not be sensitive enough to detect low protein levels. This possibility is supported by the fact that 10 patients who were WASP negative had mutations (missense and invariant splice site) expected to result in WASP expression. In this selected cohort of patients with XLT, the clinical outcome of patients who did not express WASP was not different from patients who expressed WASP. Similarly, we did not find any beneficial effect of IVIG or AB prophylaxis on overall and event-free survival or on the incidence of life-threatening infectious events. These results have to be interpreted with caution, and a possible beneficial effect of these measures cannot be ruled out because data on AB and IVIG prophylaxis were very heterogeneous about dose and duration of treatment. They might solely reflect the fact that, by definition, most patients with XLT can mount effective antibody responses and therefore do not need IVIG or AB prophylaxis. It is possible that the initiation of these prophylactic measures might have been triggered by slightly more severe disease symptoms.[33]

In this cohort of 173 patients, 108 (62%) had missense mutations in the first 4 *WAS* exons; the remaining 38% (including 11 patients with missense mutations in exons 6-12) were spread over the entire gene, including 19% in noncoding regions. This is in line with previous reports of XLT.[13-15,33] We could not detect any

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influence of the type of mutation on survival or on the incidence of specific disease-related events. A mild phenotype despite a deleterious mutation might be due to other disease-modifying genes, pathogen exposure, or somatic mosaicism caused by in vivo reversion, leading to some WASP expression and thus a milder phenotype. Reversion is an event quite frequent in WAS,[38,39] but it was not specifically analyzed in this cohort.

Forty-one patients (23.7%) had undergone splenectomy, reflecting the acceptance of splenectomy by some health care providers to reduce the risk of bleeding and thus improve quality of life in patients with XLT.[37,40] Interestingly, there was only a nonsignificant reduction of severe bleeding episodes after splenectomy, possibly because of the low overall incidence that decreased with age. However, the incidence of severe infections was significantly increased, especially in patients not receiving AB prophylaxis. These data suggest that, before splenectomy in patients with XLT, one needs to carefully weigh the pros and cons of this procedure. If performed, that is, in patients with recurrent episodes of serious bleeding, the family must understand the risk of infections and be willing to accept the need for AB prophylaxis. In addition, vaccination against pneumococci and meningococci has to be considered, given the fact that most patients with XLT can be effectively immunized.[33] The high incidence of severe infectious complications after splenectomy, including adult patients, highlights the importance of lifelong AB prophylaxis in patients with XLT who have undergone splenectomy.

The excellent overall survival rate that is close to that of the normal male population supports the perception that XLT is a mild, chronic disease and that, as a rule, patients with XLT do not require standard prophylactic interventions. Declining immune function has been observed in XLT, and defective antibody responses may require prophylactic measures such as IVIG in some patients. However, the reduced event-free survival shows substantial risks of severe, life-threatening or potentially debilitating disease-related complications. The cumulative incidence rate analysis of events showed that serious bleeding episodes were generally restricted to the first 30 years of life. In contrast, the risk of developing autoimmune disease, developing malignancy, or having a life-threatening infectious episode was rather constant throughout the patients' lifetime. The prevalence of autoimmunity is 12% in our cohort, suggesting that this complication is less common than in classic WAS whereby it was reported to be as high as 40% to 72%.[20,41,42] Interestingly, we found a significantly higher incidence of autoimmune nephropathy in Japanese patients. Similarly, the prevalence of malignancy was less in our XLT cohort (5%) than in classic WAS (13%).[20,43] Considering the higher mean age of patients with XLT compared with patients with classic WAS who have not received a transplant, these differences are even more significant.

The persistent morbidity associated with XLT might argue for HSCT as a treatment option for these patients. Given the excellent success in young children with classic WAS,[23,24] HSCT might be considered a viable option for patients with XLT if an human leukocyte antigen–identical donor can be identified. However, when discussing HSCT, which requires full conditioning in patients with WAS and patients with XLT, one needs to carefully weigh the advantage of a possible cure against the acute risks and long-term consequences of this procedure, such as risk of secondary malignancy and infertility. Thus, HSCT in XLT has to be decided on an individual patient basis. In our cohort 25 of 173 patients underwent HSCT at a median age of 7.3 years (range, 2.1-38.0 years) and 22 (88%) are alive after a median follow-up of 2.2 years (range, 0.0-12.1 years). Of note, more than half of the patients received their transplant at an age older than 5 years, when matched unrelated transplants in WAS may have a less favorable outcome.[23] Long-term studies of HSCT in patients with XLT, not available at present, are urgently needed.

Because patients with XLT may present to different medical specialists, it seems vital to raise awareness of this probably underdiagnosed or misdiagnosed condition. Although this study showed a high overall survival rate of patients with XLT, it also showed that they are at risk of life-threatening complications. By defining the natural course of XLT and recognizing the life-long medical problems that affect the prognosis and quality of life of these patients, it has become possible to select safe and effective individualized therapies for this unique set of patients with mutations of the *WAS* gene that are generally expected to be less devastating.

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Authorship

Contribution: M.H.A., B.H.B., and H.D.O. designed the study; all authors except P.P. contributed data; M.H.A., T.C.B., P.P., and H.D.O. analyzed the data; and M.H.A., T.CB., B.H.B., and H.D.O. wrote the paper.

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Correspondence: Michael H. Albert, Dr von Haunersches Kinderspital der LMU, Lindwurmstr 4, 80337 Munich, Germany; e-mail: michael.albert@med.lmu.de.

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ORIGINAL ARTICLE

Qualitative and quantitative differences in the intensity of Fas-mediated intracellular signals determine life and death in T cells

Min-Jung Shin · Jae-Hyuck Shim · Jae-Young Lee · Wook-Jin Chae · Heung-Kyu Lee · Tomohiro Morio · Jun Han Park · Eun-Ju Chang · Sang-Kyou Lee

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Abstract Fas stimulation has been reported to promote the activation and proliferation of T lymphocytes, but the intracellular signalling pathways that mediate non-apoptotic responses to Fas are poorly defined. To distinguish between the activation signalling and the death-inducing pathway downstream of Fas, we generated a novel T cell line expressing a chimeric hCD8-FasC protein and found that stimulation with the anti-CD8 antibodies induced tyrosine phosphorylation of TCR-proximal proteins, activation of Raf-1/ERK, p38 and JNK, and increased expression of CD69, Fas, and Fas ligand. Stimulation of hCD8-FasC-induced activation of an atypical NF-κB pathway, partial cleavage of caspases, and increased expression of TRAF1, $FLIP_L$ and $FLIP_S$, thereby protecting T cells from FasL-mediated apoptosis. The proliferative response transmitted through hCD8-FasC chimeric receptors was converted into death signals when cells were stimulated, resulting in increased expression of IL-2 and Nur77 and increased caspase cleavage. Surprisingly, both the enhanced expression of $FLIP_L$ and $FLIP_S$ and the complete inhibition of $FLIP_S$ expression were functionally associated with cell death induction. These findings imply that Fas is able to trigger intracellular signalling events driving both apoptosis and activation of T cells but that cell fate is determined by quantitative and qualitative differences in intracellular signalling following Fas stimulation.

Keywords Fas · Apoptosis · Activation signals · T cells

1 Introduction

Fas (CD95/APO-1), which is a member of the tumour necrosis factor receptor (TNF-R) family, plays a fundamental role in tissue homeostasis, development, and

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M.-J. Shin · J.-Y. Lee · S.-K. Lee (✉)
Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea
e-mail: sjrlee@yonsei.ac.kr

J.-H. Shim
Department of Immunology and Infectious Diseases, Harvard University, School of Public Health, Boston, MA 02116, USA

W.-J. Chae
Department of Immunology, Yale University School of Medicine, New Haven, CT 06520, USA

H.-K. Lee
Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea

T. Morio
Department of Pediatrics, Tokyo Medical and Dental University, School of Medicine, Tokyo 113-8519, Japan

J. H. Park
Department of Microbiology, Yonsei University College of Medicine, Seoul 120-752, Korea

E.-J. Chang (✉)
Department of Anatomy and Cell Biology, College of Medicine, University of Ulsan, Seoul 138-736, Korea
e-mail: ejchang@amc.seoul.kr

regulation of the immune system [1–3]. The engagement of Fas results in the formation of the death-inducing signalling complex (DISC), in which FADD binds to the cytoplasmic domain of Fas via its death domain (DD) and recruits the death effector domain (DED)-containing caspase-8 to the DISC [1, 3–5]. Pro-caspase-8 then undergoes auto-proteolysis so that it can leave the DISC and gain access to other substrates that must be proteolysed for the cell to die [6, 7]. The c-FLIP protein, which is structurally similar to caspase-8, contains DEDs but has no enzymatic activity and has been shown to have opposing functions in Fas-mediated apoptosis depending upon the level of its expression [8, 9]. In certain cells, such as hepatocytes and pancreatic β cells, Fas-mediated apoptosis requires amplification through caspase-8-mediated proteolytic activation of Bid, which is dispensable in lymphoid cells [10, 11]. Death receptor-mediated activation-induced cell death [AICD, recently proposed to be called restimulation-induced cell death (RICD)], occurs as a result of a strong activation signal and plays an important role in the deletion of autoreactive T cells in the thymus, peripheral autoreactive T cells with specificity for autoantigens, and activated T cells at the termination of an immune response [1, 4, 12]. In contrast, other studies have shown that a decline in the levels of cytokines that promote T cell survival initiates AICD by triggering the activation of the pro-apoptotic BH3-only protein Bim [10, 13]. More recently, the killing of activated T cells during the chronic immune response has been shown to require both Fas-induced apoptotic signalling and Bim [2, 4, 14].

Despite these findings, the other activities of Fas ligand (FasL)-Fas signalling, such as the induction of cellular proliferation and differentiation, remain poorly understood. It has been reported that Fas promotes the proliferation of human T lymphocytes and the maturation of dendritic cells in culture [15]. Additionally, stimulation of Fas with agonistic anti-Fas monoclonal antibodies (mAbs) has been demonstrated to accelerate liver regeneration in mice after partial hepatectomy, and this process was delayed in $Fas^{lpr/lpr}$ mutant mice [16]. In tissue culture, enzymatic inhibitors of caspase-8 or specific FADD inhibitors were found to impair T cell activation and proliferation in response to mitogenic or antigenic stimulation [17, 18]. The identities of the intracellular non-apoptotic signalling pathways triggered by Fas stimulation and the mechanisms by which the signalling molecules involved in this process are activated remain unknown.

To investigate the molecular nature of non-apoptotic signalling events induced by Fas, we generated stable Jurkat T cell transfectants expressing an hCD8-FasC fusion protein on the cell surface to enable the differentiation of Fas-mediated activation signals from cell death-inducing signals. We found that activation of the hCD8-FasC chimeric receptor by the anti-CD8 mAbs UCHT4 and OKT8 not only induced early and late activation signals leading to cell proliferation but also activated an atypical NF-κB pathway that inhibited FasL-induced cell death. Moreover, an increase in intracellular Ca^{2+} or PKC activation converted activation signals through the hCD8-FasC chimeric receptor into death signals by enhancing IL-2 and Nur77 expression. In contrast to previous results demonstrating a concentration-dependent anti-apoptotic function of c-FLIP, our data suggest that two independent signalling pathways in which the expression of both $FLIP_L$ and $FLIP_S$ is increased by phorbol 12-myristate 13-acetate (PMA) or $FLIP_S$ expression is inhibited by ionomycin (IM) may play an important role in the conversion of Fas-mediated activation to cell death. These results provide the first molecular evidence that qualitative and quantitative differences in signalling through the Fas receptor can modulate the fate of T cells, driving them toward either cell death or activation, and our study may have clinical benefits to the Fas-associated dysfunction such as self reactivity, immune dysfunction, malignant transformation [19].

2 Materials and methods

2.1 Construction of CD8 chimeric receptors

We constructed two chimeric receptors in which portions of Fas and CD8 are fused (Supplementary Fig. 1A). In the hCD8-FasTC chimera, the extracellular domain of Fas was replaced with the extracellular domain of CD8. The other chimera, hCD8-FasC, contains the extracellular and transmembrane domains of CD8 and the cytoplasmic domain of Fas. For construction of hCD8-FasC, cDNA fragments of the extracellular and transmembrane domains of human CD8 and the cytoplasmic domain of human Fas were amplified by PCR and cloned into the pcDNA3 eukaryotic expression vector at the *Xba*I, *Bgl*II and *Bam*HI restriction sites. For construction of the hCD8-FasTC construct, the PCR-amplified human CD8 extracellular domain and human Fas transmembrane and cytoplasmic domains were cloned into pcDNA3. These DNA constructs were confirmed by sequencing.

2.2 Antibodies and reagents

OKT3 and OKT8 hybridomas were purchased from the American Type Culture Collection (ATCC; Manassas, VA), and secreted antibodies were purified as previously described [20]. The following antibodies were used: anti-p-Tyr (clone 4G10), anti-p-Tyr-agarose conjugate, anti-ICE, anti-Fyn, and anti-Fas (clone ZB4) from Upstate Biotechnology (Lake Placid, NY); anti-caspase-3 and anti-c-Raf-1 from BD Transduction Laboratories (San Jose, CA); anti-Fas (clone

ZB4) from Medical and Biological Laboratories (Nagoya, Japan); anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-p38, anti-phospho-p38, anti-IκB-α, anti-phospho-IκB-α, anti-ERK-1/-2, anti-phospho-ERK-1/-2, and anti-phospho-Akt from New England Biolabs (Beverly, MA); anti-human CD8 UCHT-4 from Sigma (St. Louis, MO); anti-phospho-p38, anti-phospho-JNK, anti-Bcl-2, anti-PARP, anti-Vav, anti-Daxx, anti-ZAP70, and anti-PLC-γ from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-human Fas ligand, anti-human Fas (clone G254-274), and anti-CD69 from BD PharMingen (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA), ionomycin, bisindolylmaleimide I (BIM-I), Z-YVAD-CMK peptides, Z-DEVD-FMK peptides, Z-VAD-fmk peptides, and PD98059 were obtained from Calbiochem Inc. (San Diego, CA). Sodium salicylate was obtained from Aldrich Chemical (Milwaukee, WI). RiboQuant multi-probe RNase protection assay system was purchased from BD PharMingen (San Diego, CA). Other antibodies and chemicals used were obtained from Sigma Chemical (St. Louis, MO).

2.3 Transfection

For stable transfection, Jurkat cells (1×10^7) were washed with PBS supplemented with 0.1 M $MgCl_2$ and incubated with 25–50 μg plasmid on ice for 10 min and then electroporated using an ECM 600 electroporator (BTX Inc. Holliston, MA) at 1.2 kV and 25 μF. After pulsing, the cells were incubated in T75 culture flasks for 48 h at 37°C and plated in 96-well plates with medium containing 2.2 mg/ml geneticin (Gibco BRL, Rockville, MD). 293T cells were transiently transfected with 1 μg plasmid using LipofectAMINE$_{TM}$ Reagent (Gibco BRL, Rockville, MD) according to the manufacturer's instructions.

2.4 RT-PCR analysis and RPA assay

Total cellular RNA was extracted from the activated cells as indicated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNA was obtained using murine Moloney leukaemia virus reverse transcriptase (MMTV-RT, Gibco BRL) as previously described [21]. The cDNA was amplified using the Takara PCR amplification kit (Takara Biotechnology, Shiga, Japan), and PCRs were carried out in a PerkinElmer thermal cycler. Sequences of primer pairs specific for IL-2, β-actin, and nur77 have been previously described. These primers were purchased from Bioneer (Daejeon, Korea), and their sequences were determined in previous experiments [21]. RPA was performed using the RiboQuant multi-probe RNase protection assay system (PharMingen, San Diego, CA) following the manufacturer's instructions.

2.5 Apoptosis analyses

To analyse DNA fragmentation, cells (3×10^6) were lysed in 2× lysis buffer (200 mM HEPES, pH 7.5, 2% Triton X-100, 400 mM NaCl, 20 mM EDTA) and incubated with RNase at 37°C for 1 h. DNA was extracted with phenol, precipitated with 5 M ammonium acetate and absolute ethanol, and analysed by electrophoresis in 2% agarose.

2.6 Immunoprecipitation and western blot analysis

After incubation of 3×10^7 hCD8-FasC transfectants with the cross-linked OKT8 and UCHT4 antibodies (1 μg/1×10^6 cells) at 37°C, cells were lysed in lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 mM Na_3VO_4, 200 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 1% NP-40). Tyrosine-phosphorylated proteins were immunoprecipitated using anti-p-Tyr antibody coupled to agarose beads and washed in 1:5-diluted lysis buffer. Immunoprecipitates or total cell lysates were fractionated by SDS-PAGE and transferred to Immunobilon-P (Millipore, Bedford, MA) membranes. Membranes were incubated with primary antibodies, washed, incubated with horseradish peroxidase-conjugated secondary antibodies, and developed with ECL (Amersham, Uppsala, Sweden).

3 Results

3.1 hCD8-FasC chimeric receptor-mediated stimulation transduces activation signals but not cell death signals in T cells

To investigate the molecular nature of Fas-mediated non-apoptotic signals, two fusion constructs that express hCD8-Fas chimeric receptors were generated. One of these constructs expresses a fusion of the extracellular domain of human CD8 and the transmembrane (TM) and cytosolic domains of Fas (hCD8-FasTC), and the other expresses a fusion of the CD8 extracellular and transmembrane domains and the Fas cytosolic domain (hCD8-FasC) (Supplementary Fig. 1A). After transfection of 293T cells with hCD8-FasTC or hCD8-FasC, surface expression of each chimeric receptor was confirmed by FACS analysis (data not shown). The cells were incubated with either two immobilised anti-CD8 antibodies (OKT8 and UCHT4) or an anti-Fas antibody for 12 h or 24 h, and the levels of apoptosis were assessed by propidium iodide (PI) assays. OKT8 and UCHT4 stimulation resulted in cell death in the hCD8-FasTC transfectants but not in the hCD8-FasC transfectants (Fig. 1a). We next generated stable Jurkat transfectants expressing the hCD8-FasC chimeric receptor

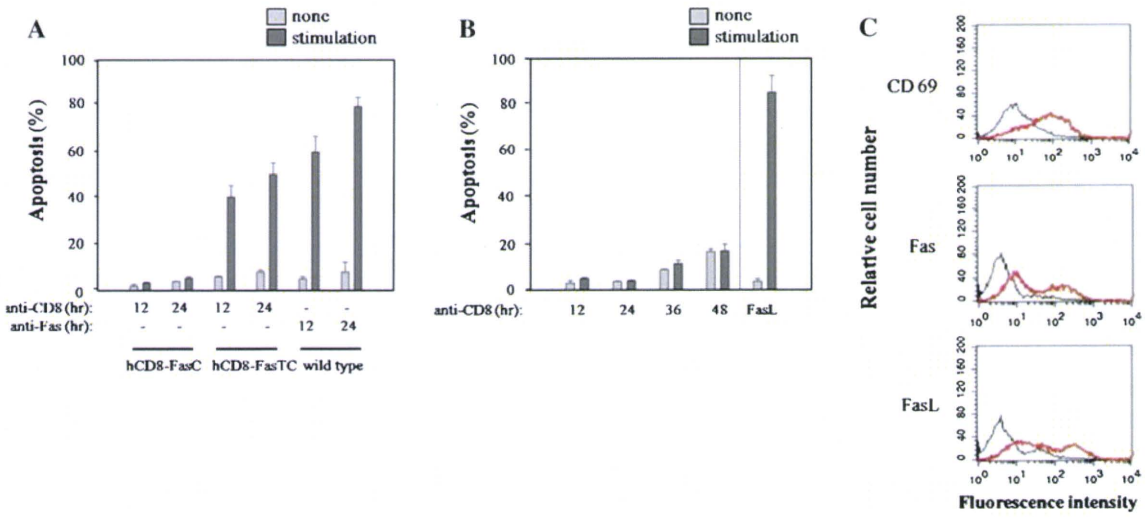

Fig. 1 Cell death was not induced by stimulation of hCD8-FasC chimeric receptor. **a** 293T cells transiently transfected with the hCD8-FasC DNA construct were stimulated with immobilised OKT8 and UCHT4 antibodies (1 μg/10^6 cells) for 12 or 24 h, and cell death was analysed. As a control, cells were stimulated with immobilised anti-Fas antibodies. **b** Stable Jurkat T cell transfectants expressing the hCD8-FasC chimeric receptor were stimulated for the indicated periods with immobilised OKT8 and UCHT4 or with NIH3T3 cells stably expressing FasL, and cell death was analysed by PI exclusion. **c** hCD8-FasC Jurkat transfectants were stimulated for 8 h with immobilised OKT8 and UCHT4 antibodies (*red lines*). Surface expression levels of CD69, Fas and FasL were analysed by flow cytometry

and confirmed the surface expression of this receptor by FACS analysis (Supplementary Fig. 1B). The hCD8-FasC Jurkat transfectants were consistently killed by FasL stably expressed on the surface of NIH3T3 cells but not by UCHT4 and OKT8 (Fig. 1b). To examine whether expression of T cell activation markers was induced in hCD8-FasC Jurkat transfectants upon stimulation with OKT8 and UCHT4, the expression levels of CD69, Fas, and FasL in anti-CD8-stimulated hCD8-FasC Jurkat cells were analysed by FACS (Fig. 1c). Activation through the hCD8-FasC chimeric receptor significantly increased the cell surface expression of CD69, Fas, and FasL. The apoptotic signal appears to be dependent on the multimerization of the chimeric receptor. Thus, apoptosis was not triggered can be the evidence which the trimeric complex was not formed in hCD8-fasC system. Or, it may not enough for inducing apoptosis for the intensity of the signal through hCD8-FasC is weaker than the wildtype trimeric complex. These results suggest that signalling through the cytoplasmic domain of Fas transduces activation signals upon stimulation, and the transmembrane domain of Fas is important for formation of the death-inducing structural conformation.

3.2 hCD8-FasC-mediated signalling activates multiple intracellular proteins related to T cell activation

To examine whether the tyrosine phosphorylation of various intracellular signalling proteins, a hallmark of T cell activation, can be induced by hCD8-FasC stimulation, hCD8-FasC transfectants were activated using UCHT4 and OKT8 antibodies. Various proteins with molecular weights of approximately 230, 210, 155, 80, 67, 64, 45, 40, 28, and 25 kDa were rapidly tyrosine phosphorylated (Supplementary Fig. 2). The resultant phosphorylated proteins were immunoprecipitated with an anti-pTyr antibody and analysed by western blotting with corresponding mAbs. Upon stimulation of hCD8-FasC, TCR-proximal proteins (Fyn, ZAP-70, Vav and PLC-γ) and MAP kinases (Erk1/2, JNK and p38) became tyrosine-phosphorylated (Fig. 2a). Surprisingly, Daxx, which is essential for Fas-mediated apoptosis, was not tyrosine-phosphorylated after hCD8-FasC-mediated activation. We next examined the kinetics of MAP kinase activation. hCD8-FasC-mediated stimulation induced prolonged phosphorylation of Raf-1, Erk1/2 and JNK, whereas phosphorylation of p38 was induced rapidly and then gradually decreased (Fig. 2b). Because PKC activation is involved in the activation of c-Raf, MEK 1/2 and Erk MAP kinase [20], we examined whether the activation of c-Raf, Erk, JNK and p38 MAP kinases through hCD8-FasC was affected by the PKC-specific inhibitor BIM-I (Fig. 2c). hCD8-FasC transfectants were pre-treated with BIM-I and then stimulated with UCHT4 and OKT8 for different periods. hCD8-FasC stimulation-induced phosphorylation of Raf-1, Erk1/2, JNK and p38 MAP kinases was dramatically suppressed by BIM-I, suggesting that activation of Erk, JNK, p38 MAP kinases following stimulation of hCD8-FasC occurs in part through a PKC-mediated signalling pathway. Because previous reports have indicated that Fas-induced JNK and p38

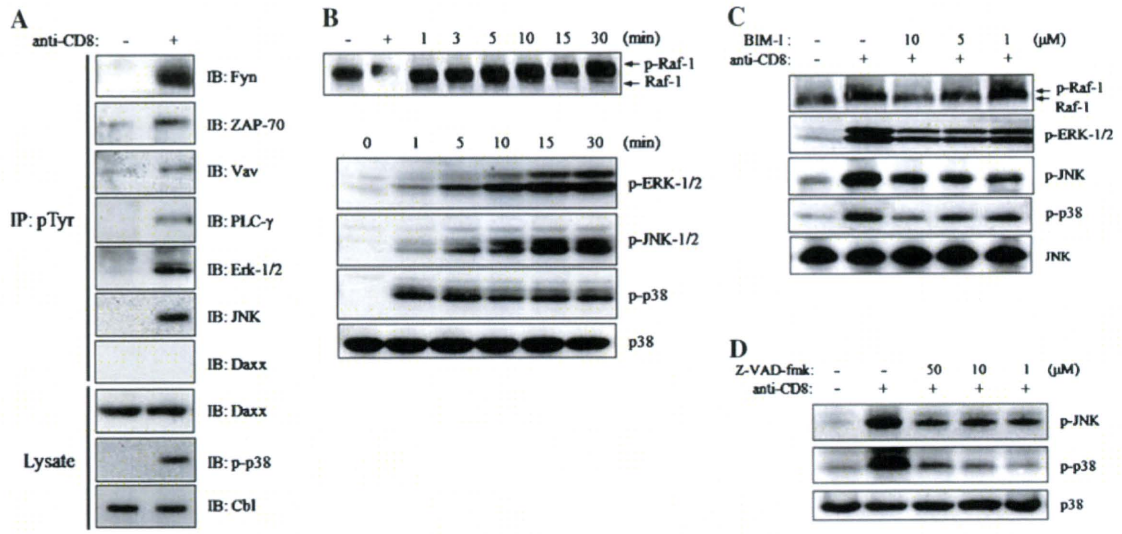

Fig. 2 hCD8-FasC-mediated signalling activates multiple intracellular proteins involved in T cell activation. **a** hCD8-FasC Jurkat transfectants were stimulated for 10 min with or without cross-linked OKT8 and UCHT4 antibodies. Cell lysates were immunoprecipitated with anti-pTyr Ab-conjugated agarose beads and immunoblotted with the indicated antibodies. **b** hCD8-FasC Jurkat transfectants were stimulated for the indicated periods with cross-linked OKT8 and UCHT4 antibodies, and cell lysates were immunoblotted with antibodies specific to phospho-Erk1/2, phospho-JNK or phospho-p38. hCD8-FasC Jurkat transfectants were treated for 1 h with or without BIM-I (1, 5, or 10 μM) (**c**) or Z-VAD-fmk (1, 10, or 50 μM) (**d**) and stimulated with OKT8 and UCHT4 antibodies for 10 min. Cell lysates were immunoblotted with antibodies specific to phospho-Erk1/2, phospho-JNK or phospho-p38

activities depend upon the activation of caspases in T lymphocytes [8, 21], we also tested whether hCD8-FasC-mediated phosphorylation of JNK and p38 MAP kinases could be blocked by caspase inhibitors (Fig. 2d). Pretreatment of hCD8-FasC transfectants with a pan-caspase inhibitor (Z-VAD-fmk) significantly suppressed phosphorylation of JNK and p38 MAP kinases in a dose-dependent manner, suggesting that JNK and p38 MAP kinase activation through hCD8-FasC is partially dependent on the activity of caspase-8, caspase-1 and caspase-3. These results imply that intracellular signals initiated by the cytoplasmic domain of Fas activate many mediators of T cell activation, such as Fyn, ZAP-70, Vav, PLC-γ and MAP kinases, but that these signals do not activate the pro-apoptotic protein Daxx, which is essential for Fas-mediated apoptosis. Additionally, we conclude that caspase and PKC activation are important for the activation of ERK and JNK/p38 MAP kinases downstream of hCD8-FasC.

3.3 NF-κB activation downstream of CD8-Fas chimeric receptor inhibits Fas-mediated apoptosis

Previous reports have implicated the NF-κB pathway in Fas-mediated cell survival; however, these findings remain controversial [15]. To determine whether hCD8-FasC-mediated activation signalling induces the activation of NF-κB, hCD8-FasC transfectants were stimulated with UCHT4 and OKT8, lysed, and immunoblotted with anti-phospho-IκB-α antibody. Activation of the T cells through hCD8-FasC immediately induced phosphorylation of IκB-α (Fig. 3a), and this hCD8-FasC-mediated NF-κB activation was dependent on caspase activity, but not on the canonical PKC-mediated NF-κB activation pathway (Fig. 3b). We next performed an RPA assay to identify anti-apoptotic proteins induced by hCD8-FasC-mediated activation (Fig. 3c). Stimulation of hCD8-FasC induced a significant increase in expression of TRAF1, but not TRAF2, and this increase in TRAF1 expression was completely inhibited by the NF-κB inhibitor sodium salicylate (Na-Sal).

To test whether NF-κB activation induced by hCD8-FasC is critical for preventing the activation of apoptotic signals in hCD8-FasC transfectants, the cells were pre-treated with Na-Sal and stimulated with immobilised UCHT4 and OKT8. Inhibition of NF-κB activation significantly increased hCD8-FasC-mediated cell death (Fig. 3d, upper panel), and cell death was accompanied by increased cleavage of caspase-3 (Fig. 3d, lower panel). We next examined whether hCD8-FasC-mediated NF-κB activation protects hCD8-FasC transfectants from anti-Fas-mediated apoptosis. The cells were pre-stimulated with immobilised UCHT4 and OKT8, incubated with NIH3T3 cells stably expressing FasL, and then analysed for cell death. Interestingly, cells stimulated through hCD8-FasC were resistant to FasL-induced apoptosis (Fig. 3e). These results suggest that hCD8-FasC-mediated activation of atypical NF-κB pathways induces the expression of TRAF1 and potent inhibitors of Fas-mediated apoptosis

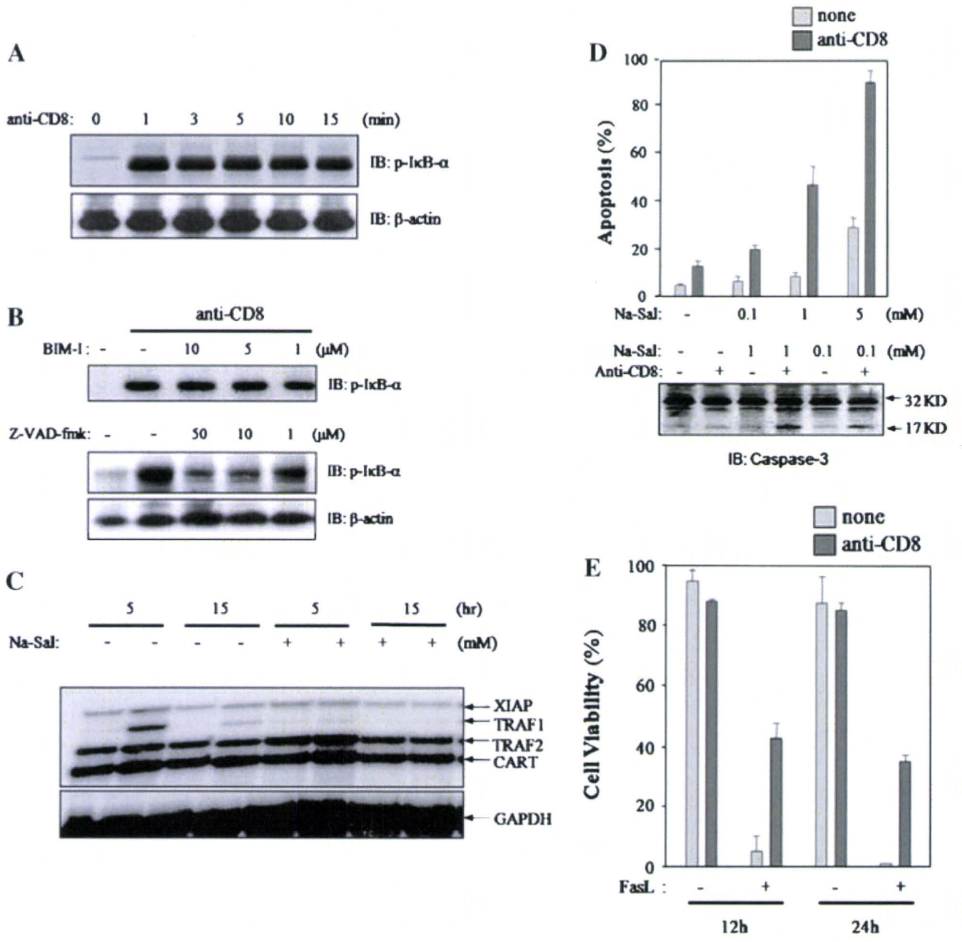

Fig. 3 hCD8-FasC-mediated NF-κB activation inhibits Fas-mediated apoptosis. **a** hCD8-FasC Jurkat transfectants were stimulated with cross-linked OKT8 and UCHT4 antibodies for the indicated periods, and cell lysates were immunoblotted with anti-phospho-IκB-α antibodies. **b** hCD8-FasC Jurkat transfectants were treated for 1 h with or without various concentrations of BIM-I (1, 5, or 10 μM) or Z-VAD-fmk (1, 10, or 50 μM) and stimulated with cross-linked OKT8 and UCHT4 antibodies for 10 min. NF-κB activation was detected by anti-phospho-IκB-α antibodies. **c** hCD8-FasC Jurkat transfectants were stimulated for 5 or 15 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 5 mM sodium salicylate. Induction of mRNAs encoding anti-apoptotic proteins was analysed by RPA. **d** hCD8-FasC Jurkat transfectants were stimulated for 12 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of sodium salicylate. Cell death was analysed by PI staining. The cleavage of caspase-3 was analysed by immunoblotting with anti-caspase-3 antibodies. **e** After hCD8-FasC Jurkat transfectants were stimulated for 12 h or 24 h with or without immobilised OKT8 and UCHT4 antibodies, cells were incubated with NIH3T3-FasL for 90 min. Cell death was analysed by PI staining

and plays a critical role in protecting cells from apoptosis induction.

3.4 Elevated PKC activation or intracellular Ca^{2+} convert hCD8-FasC-mediated activation signals into death signals

We next investigated whether hCD8-FasC-mediated T cell activation in combination with strong T cell signals such as elevated PKC activation or Ca^{2+} influx results in cell death. Treatment with PMA or IM induced a significant increase in cell death in hCD8-FasC transfectants stimulated through hCD8-FasC, as measured by DNA fragmentation (Fig. 4a). Induction of apoptosis upon stimulation through hCD8-FasC in the presence of PMA or IM was significantly blocked by cyclosporine A (CsA) or BIM-I, respectively, suggesting that the low levels of both PKC activation and Ca^{2+} influx induced by hCD8-FasC stimulation are required for hCD8-FasC-mediated apoptosis. Similar results were obtained in 293T cells transiently expressing hCD8-FasC (Fig. 4b). To confirm the effect of PKC activation or Ca^{2+} influx on Fas-induced cell death, we stimulated primary mouse T cells with two immobilised anti-Fas antibodies, which are known to induce apoptosis slowly, in the absence or presence of PMA or IM. Treatment with PMA or IM significantly enhanced Fas-mediated cell death in primary T cells (Fig. 4c). It has been demonstrated that caspase activation is required for induction of Fas-mediated apoptosis in Jurkat T

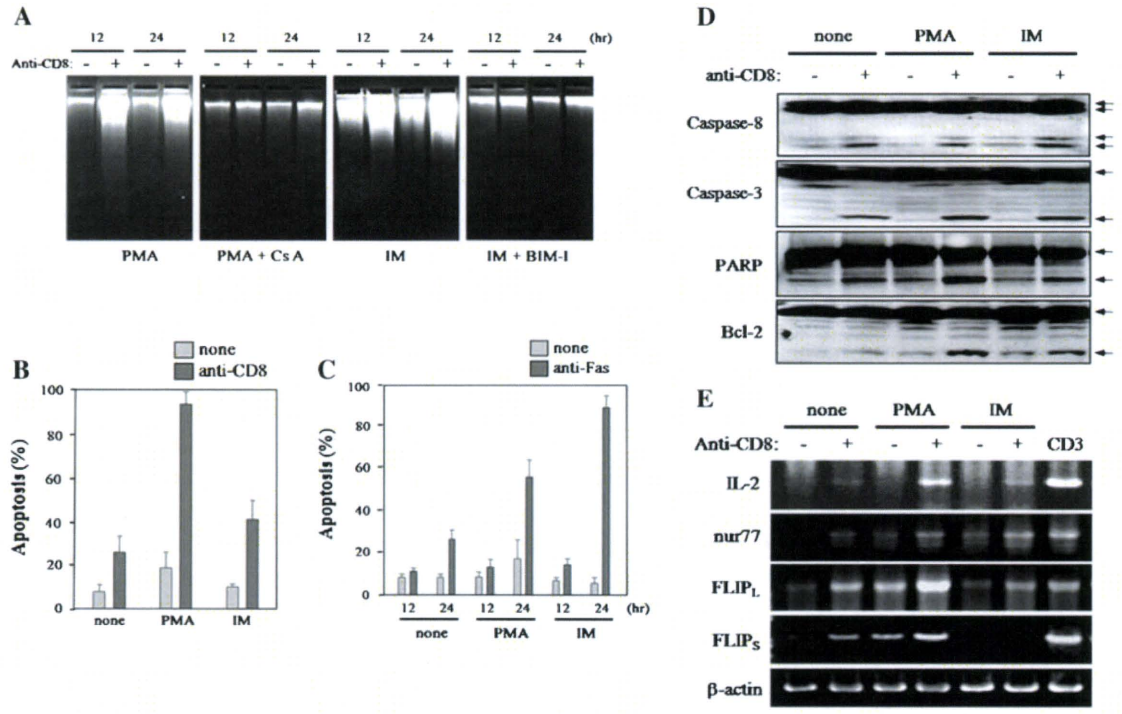

Fig. 4 PMA or ionomycin convert hCD8-FasC-mediated activation signals to death signals. **a** hCD8-FasC Jurkat transfectants were stimulated for 12 h or 24 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA, 1 ng/ml PMA and 10 μg/ml cyclosporine A, 50 nM IM, or 50 nM IM and 1 μM BIM-I. Cell death was analysed by DNA fragmentation assay. **b** 293T cells were transiently transfected with hCD8-FasC DNA and stimulated with cross-linked OKT8 and UCHT4 antibodies in the absence or presence of PMA or IM. Cell death was analysed by PI exclusion. **c** Peripheral T cells were stimulated for 12 or 24 h with two immobilised agonistic anti-Fas antibodies, ZB4 and G254-274, in the absence or presence of 5 ng/ml PMA or 100 nM IM. Cell death was analysed by PI exclusion. **d** hCD8-FasC Jurkat transfectants were stimulated for 1 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA or 50 nM IM, and cell lysates were immunoblotted with the indicated antibodies. **e** hCD8-FasC Jurkat transfectants were stimulated for 5 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA or 50 nM IM. RNA was extracted from the cells, and RT-PCR analysis was performed using primers specific for IL-2, Nur77, FLIP$_L$ and FLIP$_S$. As a positive control, cells were stimulated with immobilised OKT3 antibodies in the presence of 1 ng/ml PMA or 25 ng PMA and 200 nM IM

cells [22]. While caspase-3 cleavage and activation was substantially enhanced by hCD8-FasC stimulation and even further increased by the addition of PMA or IM, proteolytic cleavage of caspase substrates such as PARP and Bcl-2 was significantly induced by hCD8-FasC stimulation in combination with PMA, but not in combination with IM. In contrast, efficient cleavage of caspase-8 was detected following hCD8-FasC-mediated activation, and additional stimulation with PMA or IM did not enhance caspase-8 cleavage (Fig. 4d).

Stimulation of the TCR complex has been reported to induce sensitivity to cell death by increasing the expression of Fas, FasL, IL-2, and Nur77 in T cell hybridomas [23]. To determine whether hCD8-FasC-mediated apoptosis induction in the presence of PMA or IM results in increased IL-2 and Nur77 expression, hCD8-FasC transfectants were stimulated with immobilised UCHT4 and OKT8 in combination with PMA or IM, and expression of IL-2, Nur77, FLIP$_L$ and FLIP$_S$ was analysed (Fig. 4e). Although hCD8-FasC stimulation resulted in only a slight induction of Nur77 expression, both PMA and IM significantly increased Nur77 mRNA expression levels. The levels of Nur77 mRNAs induced by hCD8-FasC stimulation in combination with PMA or IM were comparable to those observed upon TCR stimulation. Stimulation of hCD8-FasC induced a substantial increase in IL-2, FLIP$_L$ and FLIP$_S$ expression that was further enhanced only by PMA; IM treatment resulted in a significant abrogation of hCD8-FasC-mediated FLIP$_S$ induction. In contrast, hCD8-FasC stimulation in the presence of either PMA or IM effectively induced cell death. The level of IL-2 induction by hCD8-FasC was not increased by IM, suggesting that the signalling pathway leading to cell death downstream of TCR stimulation may be similar to that downstream of hCD8-FasC and PKC activation but different from that downstream of hCD8-FasC and increased Ca^{2+} influx. Taken together, these results demonstrate that enhanced PKC activation or an increase in intracellular Ca^{2+} influx

can convert T cell activation signals transmitted through the cytoplasmic domain of Fas into death signals. Changes in PKC activation or intracellular Ca^{2+} influx generate distinct intracellular signalling contexts that affect cell fate after Fas stimulation by altering expression of $FLIP_L$ and $FLIP_S$, activation of caspases and subsequent cleavage of their substrates.

4 Discussion

Death receptors have been suggested to carry out several non-apoptotic functions, such as the induction of cellular activation, proliferation, differentiation, or migration, but the nature of the intracellular signalling pathways involved in these non-apoptotic functions is poorly understood. In addition to identifying the complexes formed upon death receptor stimulation, determining the exact stoichiometry of the signalling molecules involved may shed light on the molecular mechanisms driving life versus death decisions in T cells. It has been shown that Fas makes trimeric complex upon activation by ligands or antibodies. In the previous reports the proliferation of human T lymphocytes and the maturation of dendritic cells were also promoted by ligand- or agonistic mAb-mediated Fas stimulation. This process was delayed in $Fas^{lpr/lpr}$ mutant mice and deletion of Fas in T cells causes lymphopenia [15, 16]. These results suggest that stimulation-induced formation of trimeric Fas receptor can induce cell death as well as cell survival, and quantitatively or qualitatively differential signals through Fas receptor may determine the cell fate to death or survival. In this study, we generated a cell line system to differentiate the Fas-mediated nonapoptotic signalling pathway from the death-inducing pathway. Stimulation of an hCD8-FasC chimeric receptor, which contains the cytoplasmic domain of Fas, may induce T cell proliferative signals by activating key TCR-proximal proteins, such as Fyn, Zap-70, Vav, PLC-γ, Raf-1/ERK and p38/JNK MAP kinases, and increasing the expression of CD69, Fas, and Fas ligand. In contrast to the Fas-mediated death signalling pathway, hCD8-FasC-mediated signalling did not activate Daxx/Ask-1, which is an essential factor in cell death. We also found that hCD8-FasC-mediated activation of Raf, ERK and JNK/p38 MAP kinases is dependent on PKC and caspase activation. Importantly, the T cell activation signals downstream of hCD8-FasC-induced NF-κB activation in a caspase-dependent but not PKC-dependent manner, suggesting that these signals are mediated by an atypical NF-κB activation pathway. The low levels of caspase-8 and caspase-3 activation and subsequent NF-κB activation induced the expression of low levels of anti-apoptotic proteins such as TRAF1, Nur77, $FLIP_L$ and $FLIP_S$, which are important for maintaining hCD8-FasC-mediated signals as activation signals, but not for cell death signal.

In contrast, others have reported that the transmembrane and cytoplasmic domains of Fas in other chimeric receptors, such as CD40-Fas, TNFR1-Fas, CD44-Fas, mainly induce cell death upon activation by their cognate antibodies [24]. In accordance with these results, another chimeric receptor generated in our study, hCD8-FasTC, containing both the transmembrane and the cytoplasmic domain of Fas, was found to induce cell death similarly to the above death-inducing chimeric receptors. Together, these results suggest that the discrete mechanisms of Fas receptor stimulation by its cognate ligand and the presence of the transmembrane domain of Fas receptor are important in forming the unique conformation required to transmit the signal for cell death.

Surprisingly, activation signals downstream of hCD8-FasC or Fas receptor were converted into death signals in the presence of elevated PKC activation or Ca^{2+} influx; these death signals were reflected in increased expression of Nur77 and cleavage of caspase-3 and its substrates. The potential of PMA or IM to convert hCD8-FasC-mediated activation into a cell death signal was completely inhibited by CsA or a PKC inhibitor, respectively, demonstrating that balanced and low levels of both PKC activation and Ca^{2+} influx are required for hCD8-FasC-induced T cell activation, and the enhancement of either of these two signals can alter the intracellular activation status of T cells, resulting in cell death.

The essential roles of caspase-8, NF-κB activation and FLIP expression in the modulation of Fas-mediated cell death have been demonstrated in several studies and are widely accepted, but the function of these proteins in the activation of non-apoptotic signalling pathways is still controversial [3, 4]. Consistent with previous findings, our study demonstrated that low levels of cleavage and activation of caspase-8, caspase-3 and caspase-3 substrates were induced by hCD8-FasC-mediated activation signals, and treatment with PMA but not IM enhanced this caspase cleavage. Low levels of IL-2, Nur77, $FLIP_L$ and $FLIP_S$ expression were induced by stimulation of hCD8-FasC. PMA treatment substantially enhanced the expression of these proteins, leading to cell death, but IM treatment did not affect IL-2 expression and significantly inhibited induction of $FLIP_L$ and $FLIP_S$ expression. These results suggest that at least two independent signalling pathways, a PKC-mediated pathway and a Ca^{2+} influx-dependent pathway, may be involved in the conversion of Fas-mediated activation signals to death signals, and $FLIP_L$ and $FLIP_S$ may play different roles in driving cells toward apoptosis or survival. Recently, two N-terminal cleavage products of cFLIP, p43-FLIP and p22-FLIP, have been reported to play an important role in NF-κB activation. We are currently analysing the function of these two proteins in the modulation of Fas-mediated activation and apoptosis in our hCD8-FasC transfectants.

Several studies demonstrated that T cell proliferation induced by suboptimal anti-CD3 stimulation is enhanced when Fas is triggered [15]. Also, deletion of CD95 in T cells causes lymphopenia in mice, suggesting that CD95 expression by T cell is required for the survival, proliferation and activation. FADD, caspase-8, and c-FILP are known to link Fas to nonapoptotic pathways. The non apoptotic outcomes may result in response to particular circumstances as inhibition at the receptor level, inappropriate concentrations of caspase-8/caspase-10 or of downstream proapoptotic proteins such as Bax, upregulation of protective molecules, or activation of protective pathways. These studies could tell that the differences of specific microenvironments might decide the fate of the Fas signal rather than the indispensability of transmembrane domain or formation of trimeric structure of Fas.

Taken together, our findings suggest that Fas-mediated signals may be capable of inducing both cell death and activation, that the stimulation of Fas receptor in distinct intercellular contexts results in the formation of a discrete signalling conformation, and that the ensuing quantitative and qualitative differences in the intracellular signalosome are critical in determining the outcome of Fas-mediated signalling. Additionally, the differences of specific microenvironment around T cells expressing Fas, the expression level, localisation, extent of activation and modification of initiator and executor caspases and their substrates and adaptor proteins may be also essential in determining the fate of T cells after interaction of Fas and FasL rather than formation of trimeric complex of Fas or indispensability of its transmembrane domain. From clinical point of view, our results may lead to better understanding of the pathogenesis of immunological disease such as autoimmune lymphoproliferative syndrome (ALPS) to the development of measures to manipulate Fas-mediated signal in the patients [25].

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LETTERS

IκBζ regulates T_H17 development by cooperating with ROR nuclear receptors

Kazuo Okamoto[1,2,3], Yoshiko Iwai[4], Masatsugu Oh-hora[1,2], Masahiro Yamamoto[5], Tomohiro Morio[6], Kazuhiro Aoki[7], Keiichi Ohya[7], Anton M. Jetten[8], Shizuo Akira[9], Tatsushi Muta[10] & Hiroshi Takayanagi[1,2,3]

Interleukin (IL)-17-producing helper T (T_H17) cells are a distinct T-cell subset characterized by its pathological role in autoimmune diseases[1–3]. IL-6 and transforming growth factor-β (TGF-β) induce T_H17 development, in which the orphan nuclear receptors, RORγt and RORα, have an indispensable role[4–6]. However, in the absence of IL-6 and TGF-β, the ectopic expression of RORγt or RORα leads to only a modest IL-17 production[5,7,8]. Here we identify a nuclear IκB family member, IκBζ (encoded by the *Nfkbiz* gene), as a transcription factor required for T_H17 development in mice. The ectopic expression of IκBζ in naive $CD4^+$ T cells together with RORγt or RORα potently induces T_H17 development, even in the absence of IL-6 and TGF-β. Notably, $Nfkbiz^{-/-}$ mice have a defect in T_H17 development and a resistance to experimental autoimmune encephalomyelitis (EAE). The T-cell-intrinsic function of IκBζ was clearly demonstrated by the resistance to EAE of the $Rag2^{-/-}$ mice into which $Nfkbiz^{-/-}$ $CD4^+$ T cells were transferred. In cooperation with RORγt and RORα, IκBζ enhances *Il17a* expression by binding directly to the regulatory region of the *Il17a* gene. This study provides evidence for the transcriptional mechanisms underlying T_H17 development and points to a molecular basis for a novel therapeutic strategy against autoimmune disease.

IκBζ is a nuclear protein homologous to Bcl3, which interacts with the NF-κB subunit via the ankyrin repeat domain (ARD)[9,10]. In macrophages, IκBζ induced by Toll-like receptor (TLR) stimulation is essential for the induction of a subset of secondary response genes, including *Il6* (refs 11, 12). However, the function of IκBζ in other cell types has not been elucidated.

We explored the expression of IκBζ in helper T-cell subsets and found that IκBζ was most highly expressed in T_H17 cells (Fig. 1a). Thus, we evaluated the involvement of IκBζ in EAE, which is a model of T_H17 cell-mediated autoimmune disease[1]. After myelin oligodendrocyte glycoprotein (MOG) immunization, wild-type mice developed severe paralytic symptoms, whereas $Nfkbiz^{-/-}$ mice exhibited almost no neuronal deficit (Fig. 1b). Histopathological analyses showed inflammatory cell infiltration and demyelination in the spinal cord of the wild-type mice, but not the $Nfkbiz^{-/-}$ mice (Fig. 1c). IL-17 production was reduced in the spleen and lymph node cells from immunized $Nfkbiz^{-/-}$ mice, but interferon-γ (IFN-γ) production was normal (Fig. 1d and Supplementary Fig. 1). These results indicate that $Nfkbiz^{-/-}$ mice have a defect in T_H17 development.

IκBζ expression was also detected in dendritic cells (Fig. 1a), which produce the inflammatory cytokines required for T_H17 development[1,13]. To exclude the possibility that impaired T_H17 development in $Nfkbiz^{-/-}$ mice is caused by a defect in dendritic cells, we evaluated inflammatory cytokine expression in dendritic cells. We confirmed normal production of tumour necrosis factor-α (TNF-α) and expression of CD40 and CD86 in $Nfkbiz^{-/-}$ dendritic cells after TLR stimulation (Supplementary Fig. 2 and data not shown). TLR ligand-induced production of IL-6 was partially suppressed in $Nfkbiz^{-/-}$ dendritic cells (Supplementary Fig. 2), but a co-culture of naive $CD4^+$ T cells and dendritic cells indicated that $Nfkbiz^{-/-}$ dendritic cells were able to support T_H17 development normally (Fig. 1e, f). However, T_H17 development from $Nfkbiz^{-/-}$ naive $CD4^+$ T cells was markedly impaired regardless of the origin of the co-cultured dendritic cells (Fig. 1e, f).

To demonstrate the $CD4^+$ T-cell-intrinsic function of IκBζ *in vivo*, we transferred wild-type or $Nfkbiz^{-/-}$ $CD4^+$ T cells into $Rag2^{-/-}$ mice (Supplementary Fig. 3), and immunized them with MOG peptide. The mice transferred with wild-type $CD4^+$ T cells developed severe EAE, whereas the mice transferred with $Nfkbiz^{-/-}$ $CD4^+$ T cells had only minimal symptoms (Fig. 1g, h). We observed no significant difference in proliferation between wild-type and $Nfkbiz^{-/-}$ $CD4^+$ T cells (Supplementary Fig. 4). The frequencies of IFN-γ^-IL-17^+ and IFN-γ^+IL-17^+ T cells, but not IFN-γ^+IL-17^- T cells, were much lower in the mice that received $Nfkbiz^{-/-}$ $CD4^+$ T cells than wild-type $CD4^+$ T cells, even at early time points after MOG immunization (Fig. 1i). Collectively, $Nfkbiz^{-/-}$ $CD4^+$ T cells have an intrinsic defect in their ability to differentiate into T_H17 cells, resulting in a low sensitivity to EAE.

When activated with anti-CD3 and anti-CD28 under T_H1- and T_H2-polarizing conditions, $Nfkbiz^{-/-}$ naive $CD4^+$ T cells normally produced IFN-γ and IL-4, respectively (Fig. 2a). On activation by the combination of IL-6 and TGF-β (the T_H17-polarizing conditions), IL-17 production in $Nfkbiz^{-/-}$ T cells was significantly reduced compared with wild-type T cells, even when we added IL-23, IL-1α, TNF-α or replaced IL-6 with IL-21 (Fig. 2a, b). The expression of *Il17f*, *Il21*, *Il23r* and *Il22* messenger RNA was much lower in $Nfkbiz^{-/-}$ T cells than in wild-type T cells (Fig. 2c). The expression of *Rorc* (encoding RORγ) and *Rora* (encoding RORα) mRNA was comparable between wild-type and $Nfkbiz^{-/-}$ T cells (Fig. 2d). The mRNA expression of *Runx1*, the aryl hydrocarbon receptor (*Ahr*), *Irf4*, *Socs3* and *Batf*,

[1]Department of Cell Signaling, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, [2]Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, [3]Japan Science and Technology Agency (JST), ERATO, Takayanagi Osteonetwork Project, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8549, Japan. [4]Medical Top Track Program, Medical Research Institute, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8510, Japan. [5]Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, and WPI Immunology Frontier Research Center, Osaka University, 2-2, Yamada-oka, Suita, Osaka 565-0871, Japan. [6]Department of Pediatrics and Developmental Biology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8519, Japan. [7]Department of Hard Tissue Engineering (Pharmacology), Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8549, Japan. [8]Cell Biology Section, Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, 111 T.W. Alexander Drive Research Triangle Park, North Carolina 27709, USA. [9]Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, Yamada-oka 3-1, Suita, Osaka 565-0871, Japan. [10]Laboratory of Cell Recognition and Response, Graduate School of Life Sciences, Tohoku University, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan.

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