松村 到, 金倉 譲.	イマチニブ耐性・不耐容の慢性骨髄性白血病に対する治療戦略.	総合臨床	57	666-674	2008
松村 到, 金倉 譲.	MPDに対する分子標的療法.	カレントテラピー	26	56-60	2008
松村 到, 金倉 譲.	今後の分子標的療法候補薬.	骨髄異形成症候群(MDS)の基礎と臨床		264-273	2008
松村 到, 金倉 譲.	急性骨髄性白血病(AML)における白血病幹細胞.	医学のあゆみ	102	pp5-9	2008
松村 到, 金倉 譲.	好酸球増加症に対するヒト化抗IL-5中和抗体mepolizumabの治療効果.	内科	33(3)	1021-1022	2008
松村 到, 金倉 譲.	急性骨髄性白血病の分類と診断、治療指針.	血液フロンティア	18	29-41	2008
松村 到, 金倉 譲.	Imatinib耐性の原因と対策.	分子細胞治療	7	23-30	2008
西村純一, 金倉 譲.	造血器疾患治療の新しい潮流 発作性夜間血色素尿症の治療.	Mebio	25	37-43	2008
西村純一, 金倉 譲.	貧血 -最新の基礎と臨床- 基礎編 貧血の分子病態 -各論- 発作性夜間ヘモグロビン尿症(PNH).	日本臨床	66	490-496	2008
西村純一, 金倉 譲.	II.血液・造血器疾患を深く学ぼう A.赤血球系疾患 発作性夜間ヘモグロビン尿症.	血液・造血器疾患学		146-149	2008
増田有美子, 野島順三, 岩谷良則, 末久悦次, 二日市良彰, 日高 洋, 金倉 譲.	全血法によるエンドトキシン誘発単球表面組織因子発現量の測定と臨床応用.	日本検査血液学会雑誌	9	23-30	2008

研究成果の刊行に関する一覧表(和文)(平成21年度)

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
丹羽明、中畑龍俊	I. 造血幹細胞　I．iPS細胞からの造血分化	Annual Review　血液	2009	1-8	2009
澤田明久、井上雅美、近藤統、木本富子、山田佳世、中山雅弘、桑江優子、西川正則、大川洋二、井田孔明、徳田桐子、真部淳、土屋邦彦、奥山宏臣、窪田昭男、川原央好、長谷川利路、米田光宏、竹本理、山田淳二、川端秀彦、田村太資、木内恵子、平野慎也、宇野誠、竹下泰史、石原卓、岡村隆行、坂田尚己、水谷修紀、中畑龍俊、迫正廣、多和昭雄、尾路祐介、坪井昭博、小山真穂、岡芳弘、安井昌博、杉山治夫、河敬世	小児がんに対するWT1ペプチドによるワクチン療法	日本小児がん学会雑誌	46-1	6-16	2009
深尾大輔、加藤格、梅田雄嗣、徳舛麻友、瓜生久美子、馬場志郎、松原央、渡邉健一郎、土井拓、足立壮一、中畑龍俊、水嶋康浩、片岡昭浩、若園吉裕	感染性心内膜炎合併後に安全に非血縁臍帯血移植を施行し得た乳児白血病の１例	日本血液学会雑誌	23-2	126-130	2009
河井昌彦、中畑龍俊	プロトロンビン時間を重視した早期新生児のDIC診断基準	日本産婦人科新生児血液学会誌	18-2	85-90	2009
長井静世、依藤亨、土井拓、河井昌彦、百井亨、岡本晋弥、土井隆一郎、中本裕士、増江道哉、加古伸雄、岡本浩之、加藤英治、長沖優子、上本伸二、中畑龍俊	集学的アプローチにより腫瘍核出術をしえた局所型先天性高インスリン血症	日本小児科学会雑誌	113-5	838-842	2009
伊藤仁也、中畑龍俊	対外増幅造血細胞移植	医学のあゆみ	1229-9	786-792	2009
徳舛麻友、松原央、瓜生久美子、加藤格、梅田雄嗣、渡邉健一郎、足立壮一、仲俣岳晴、中山富貴、坪山直生、戸口田淳也、中畑龍俊	京大病院にて治療を行なった6歳以下発症の骨肉腫4例の検討	小児がん	46-2	195-200	2009
堀部敬三、土田昌宏、鶴澤正仁、中畑龍俊	わが国の小児造血器腫瘍診療施設の実態	日本小児科学会雑誌	113-1	105-111	2009
中畑龍俊	さい帯血造血幹細胞の発見からさい帯血移植へ	日本さい帯血バンクネットワーク設立10周年記念『そして明日から』		26-27	2009
瓜生久美子、松原央、加藤格、徳舛麻友、梅田雄嗣、今井剛、渡邉健一郎、足立壮一、中畑龍俊	小児T細胞型急性リンパ性白血病に合併したgroove膵炎の１例	日本小児血液学会誌	23-3	209-212	2009
中畑龍俊	疾患特異的iPS細胞	学術の動向	14-9	78-83	2009

中畑龍俊	インフォームド・コンセント2－システムとしての対応．（特集　医療事故とリスクマネジメント）	小児科診療	72-10	1793-1800	2009
中畑龍俊	疾患特異的iPS細胞	MSD メディカル・サイエンス・ダイジェスト 10月臨時増刊号	35	12-459	2009
中畑龍俊	さまざまな幹細胞を用いた今後の再生医療	血液フロンティア 11月号	19-11	17-18	2009
中畑龍俊	疾患特異的iPS細胞の医療応用	再生医療	8-4	7	2009
16．河井昌彦、磯目賢一、三宅史子、岩永甲午郎、松倉崇、柴田実、丹羽房子、中畑龍俊	ＳＧＡ児のメタボリック・シンドロームのリスクについての検討―ＳＧＡ児の高血圧は幼児期から始まる？	日本周産期新生児医学会雑誌	45(4)	1274-1275	2009
万木紀美子、前川　平	Ⅳ．血液製剤．2）輸血療法の実際．	綜合臨床 第58巻増刊号「今すぐに役立つ 輸液ガイドブック」	58	390-398	2009
八尾尚幸、芦原英司、前川　平	支持療法　ー輸血・成分輸血、Epo, G-CSFなど ー．特集「骨髄性白血病　ー　病因・治療研究の進歩」	日本臨床	67(10)	1951-1957	2009
西川昭子、村山敏典、前川　平	産業界のためのガイダンス ヒトの細胞，組織，および細胞・組織製剤－(HCT/P)に関する規制　小規模事業所の準拠ガイド．	臨床評価	36	183-196	2009
西川昭子、村山敏典、前川　平	産業界のためのガイダンス ヒトの細胞，組織，および細胞・組織製剤―(HCT/P)のドナーの適格性判断．	臨床評価	36	197-256	2009
西川昭子、村山敏典、前川　平	産業界のためのガイダンス―第１相研究新薬のためのCGMP (Guidance for Industry－CGMP for Phase 1 Investigational Drugs)．	臨床評価	36	257-273	2009
前川　平	開発段階に応じたGMP規制(stepwise approach, フェーズ1 GMP)の重要性―アカデミアにおけるトランスレーショナルリサーチ推進のために―．	臨床評価	36	274	2009
松村　到，金倉　譲	造血の分子機構．	内科学書		8-9	2009
松村　到，赤司浩一，薄井紀子，神田善伸，宮崎泰司	慢性骨髄性白血病治療の現状と病態に基づいた今後の治療．	Trends in Hematological Malignancies	1	8-17	2009
水木満佐央，金倉　譲	慢性骨髄性白血病．	入門腫瘍内科学		244-246	2009
水木満佐央，金倉　譲	肥満細胞症．	WHO分類第4版による白血病・リンパ系腫瘍の病態学		47-55	2009
織谷健司，金倉　譲	Bリンパ球初期分化と制御機構．	臨床免疫・アレルギー科	51	66-71	2009
織谷健司，金倉　譲	ダサチニブ(スプリセル)．	腫瘍内科	4	79-84	2009
柴山浩彦，金倉　譲	ヘアリーセル白血病(HCL)．	造血器腫瘍アトラス		309-313	2009
横田貴史，織谷健司，金倉　譲	新たな造血幹細胞マーカーESAM．	血液・腫瘍科	59	209-216	2009

田中宏和, 松村　到, 金倉　譲	白血病幹細胞研究の動向.	Biotherapy	23	364-370	2009
鹿村真之, 伊藤仁也, 大隈一興, 関根暉彬	リンパ球活性化培養中の細胞表面抗原の経時的変化	Biotherapy	23	257-262	2009
伊藤仁也, 中畑龍俊	体外増幅造血細胞移植	医学のあゆみ	Vol.229 No.9	786-792	2009

研究成果の刊行に関する一覧表(和文)（平成22年度）

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
中畑龍俊	造血因子と臨床応用	臨床検査	第54巻 第6号	623-629	2010
中畑龍俊	iPS細胞と遺伝性疾患（特集　臨床遺伝学の進歩と日常診療．遺伝性疾患の新しい治療と今後期待される治療研究）	日本医師会雑誌	第139巻 第3号	632-634	2010
河井昌彦、三宅史子、岩永甲午郎、松倉崇、丹羽房子、中畑龍俊	早期新生児期のDICパラメータの出生体重・日齢に伴う変化についての検討.	日本産婦人科・ 新生児血液学会誌	第19巻 第2号	33-36	2010
務中達也、叶井正樹、木村晋也、芦原英司、阿部浩久、庄司習一、前川　平	細胞解析チップ―生体内のがん細胞の挙動研究を目指して―	島津評論	66	141-149	2010
村松裕子、木村晋也、一戸辰夫、芦原英司、石川隆之、前川 平、内山 卓	造血幹細胞移植における血縁ドナーのための専門外来	日本輸血細胞治療学会雑誌	56	68-71	2010
金倉　譲，松村　到	血液疾患（1.総論、2.赤血球異常、3.白血球異常）	口腔外科学 （白砂兼光，古郷幹彦編）	第11章	430-447	2010
金倉　譲	骨髄増殖性疾患の概念	臨床検査		227-233	2010
金倉　譲	総論：白血病治療の進歩	BIO Clinica		16-17	2010
永井謙一、橋本尚子、伊藤仁也、松下章子、下地園子、木村隆治、井上大地、森美奈子、永井雄也、田淵淑江、柳田宗之、高橋隆幸	非血縁子通津伊移植後の再発に対する臍帯血移植後に、第1ドナーリンパ球による移植片対白血病効果が認められたＴリンパ芽球性リンパ腫	臨床血液	第51巻 第6号	別冊	2010

Ⅴ．研究成果の刊行物・印刷物

Pediatr Transplantation 2008: 12: 862–867

Pediatric Transplantation
DOI: 10.1111/j.1399-3046.2008.00931.x

A conditioning regimen of busulfan, fludarabine, and melphalan for allogeneic stem cell transplantation in children with juvenile myelomonocytic leukemia

Yabe M, Sako M, Yabe H, Osugi Y, Kurosawa H, Nara T, Tokuyama M, Adachi S, Kobayashi C, Yanagimachi M, Ohtsuka Y, Nakazawa Y, Ogawa C, Manabe A, Kojima S, Nakahata T. A conditioning regimen of busulfan, fludarabine, and melphalan for allogeneic stem cell transplantation in children with juvenile myelomonocytic leukemia.
Pediatr Transplantation 2008: 12: 862–867. © 2008 Wiley Periodicals, Inc.

Abstract: A pilot study was undertaken using a myeloablative conditioning with fludarabine, busulfan, and melphalan to improve the outcome of HSCT in 10 children, aged six months to six yr, with JMML. All patients were conditioned with oral busulfan (560 mg/m^2), fludarabine (120 mg/m^2), and melphalan (180–210 mg/m^2) prior to HSCT, and received stem cells from bone marrow in seven cases, and from cord blood in three cases. Engraftment was documented in eight patients, whereas graft failure occurred in two, one of whom had received HLA-mismatched cord blood and other had received bone marrow from HLA-mismatched mother. Three patients, including two in who graft failure had occurred, relapsed. Five patients developed acute GVHD and two developed chronic GVHD. Seven patients are alive and in remission 27–69 months after transplantation. Thus, our study showed that HSCT following conditioning with fludarabine, busulfan, and melphalan was well tolerated and appeared to be effective for JMML.

Miharu Yabe[1], Masahiro Sako[2], Hiromasa Yabe[1], Yuko Osugi[2], Hidemitsu Kurosawa[3], Taemi Nara[4], Mika Tokuyama[5], Souichi Adachi[6], Chie Kobayashi[7], Masakatsu Yanagimachi[8], Yoshitoshi Ohtsuka[9], Yozo Nakazawa[10], Chitose Ogawa[11], Atsushi Manabe[11], Seiji Kojima[12] and Tatsutoshi Nakahata[6] for a Japanese Childhood MDS Study Group

[1]Department of Cell Transplantation, Tokai University School of Medicine, Kanagawa, Japan, [2]Division of Pediatrics, Osaka City General Hospital, Osaka, Japan, [3]Department of Pediatrics, Dokkyo Medical University, Tochigi, Japan, [4]Division of Hematology and Oncology, Shizuoka Children's Hospital, Shizuoka, Japan, [5]Department of Pediatrics, Toho University School of Medicine, Tokyo, Japan, [6]Department of Pediatrics, Kyoto University School of Medicine, Kyoto, Japan, [7]Division of Pediatrics, Ibaraki Children's Hospital, Ibaraki, Japan, [8]Department of Pediatrics, Yokohama City University School of Medicine, Kanagawa, Japan, [9]Department of Pediatrics, Hyogo Medical University, Hyogo, Japan, [10]Department of Pediatrics, Shinshu University School of Medicine, Nagano, Japan, [11]Department of Pediatrics, St. Luke's International Hospital, Tokyo, Japan, [12]Department of Pediatrics, Nagoya University School of Medicine, Aichi, Japan

Key words: juvenile myelomonocytic leukemia – hematopoietic stem cell transplantation – fludarabine-containing regimen

Miharu Yabe, Department of Cell Transplantation, Tokai University School of Medicine, Shimokasuya 143, Isehara, Kanagawa 259-1193, Japan
Tel.: +81 463 93 1121
Fax: +81 463 93 8607
E-mail: miharu@is.icc.u-tokai.ac.jp

Accepted for publication 28 January 2008

Abbreviations: AML, acute myelogenous leukemia; BMT, bone marrow transplantation; CBT, cord blood transplantation; CR, complete remission; Cs-A, cyclosporin A; EBMT, European Blood and Marrow Transplantation; EWOG, European Working Group on Childhood; FISH, fluorescence *in situ* hybridization; FLAG, fludarabine, cytarabine, and granulocytes; G-CSF, granulocytes colony stimulating factor; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; JMML, juvenile myelomonocytic leukemia; MDS, myelodysplastic syndrome; MTX, methotrexate; RRT, regimen-related toxicity; STR, short tandem repeats; TBI, total body irradiation.

JMML is a rare hematologic malignancy of early childhood, which runs an aggressive clinical course (1). Allogeneic HSCT is presently the only curative treatment available for JMML (2). Some studies have reported that a graft-vs.-leukemia effect seems to play an essential role in HSCT for JMML because the development of chronic GVHD protects the patients against the risk of disease relapse (2, 3). Until recently, most studies published on the results of HSCT in patients with JMML had been performed on a limited number of patients conditioned with heterogeneous regimens. The EWOG-MDS/EBMT group reported the outcome of JMML in 100 children, who were given HSCT after a homogenous preparative regimen consisting of three alkylating agents: busulfan, cyclophosphamide, and melphalan, without TBI (4). These results, when compared, were favorable to those published previously, curing approximately 50% of patients with JMML. In addition, the outcome of HSCT recipients who received transplantation from unrelated donor was comparable to that of children who had received transplants from HLA-identical siblings. Although allogeneic HSCT for JMML has been shown to improve outcome, leukemia recurrence has been represented as the main cause of treatment failure after HSCT in JMML patients (5, 6). In this report, we describe the outcome of 10 children with JMML, who were given unmanipulated HSCT after a uniform preparative regimen comprising oral busulfan, fludarabine, and melphalan.

Patients and methods

Patients

Ten consecutive patients with primary JMML underwent HSCT after being given conditioning with fludarabine, busulfan, and melphalan between June 2001 and November 2005. The patients in this study were diagnosed as suffering from JMML according to previously published criteria (7, 8). The patient characteristics at the time of diagnosis and transplantation are listed in Tables 1 and 2, respectively. Karyotypic abnormality was detected in one patient (no. 7), who was diagnosed with monosomy-7, and chemotherapy designed for AML was employed prior to transplantation in this patient. The remaining patients were treated with various regimens. Low-intensity chemotherapy was based on the use of mercaptopurine or low-dose cytosine arabinoside. Splenectomy before HSCT was performed in one patient,

Table 1. Patient data at diagnosis

No.	Sex	Age (yr)	WBC count ($\times 10^9$/L)	Platelets ($\times 10^9$/L)	HbF (%)	Cytogenetics	Mutations
1	M	0.1	97.0	23.0	60.0	46,XY	N.T
2	F	3.1	19.0	26.0	55.0	46,XX	PTPN11
3	M	5.4	9.6	13.0	43.9	46,XY	NRAS
4	F	0.3	37.2	1.0	19.5	46,XX	N.T
5	M	1.6	56.6	63.0	62.3	46,XY	N.T
6	M	2.4	14.3	33.0	43.3	46,XY	N.T
7	F	0.6	23.8	102.0	9.2	46,XX, -7,+der(?)t(?;7)(?;q11)	N.T
8	M	1.0	53.1	1.0	5.2	46,XY	N.T
9	F	0.9	61.0	12.0	50.4	46,XX	NRAS
10	M	3.7	56.6	10.0	26.4	46,XY	PTPN11

F, female; M, male; WBC, white blood cell; HbF, fetal hemoglobin.

Table 2. Patient characteristics at transplantation

No.	Age (yr)	WBC count ($\times 10^9$/L)	BM blast (%)	Spleen size (cm)	Previous therapy	Interval to HSCT (months)
1	0.5	9.7	3.6	5	6MP	5
2	3.7	1.0	60.9	12	6MP, VP, MIT, low-dose CA, splenic irradiation 600 cGy	6
3	6.8	6.2	5.4	11.5	6MP	16
4	2.2	15.0	9.0	Splenectomized	6MP, low-dose CA, splenectomy	22
5	1.9	10.1	10.0	6	6MP	4
6	2.8	4.7	4.0	3	6MP	4
7	2.2	2.6	5.0	15	VP + CA (AML protocol)	19
8	1.6	8.9	1.4	Not palpable	6MP, PSL	7
9	1.2	1.0	6.0	6	6MP, PSL, VP	4
10	3.9	5.0	2.6	15	6MP, low-dose CA, splenic irradiation 600 cGy	3

F, female; M, male; WBC, white blood cell; BM, bone marrow; HSCT, hematopoietic stem cell transplantation; 6MP, mercaptopurine; VP, etoposide; MIT, mitoxantrone; CA, cytosine arabinoside; AML, acute myelogenous leukemia; PSL, prednisolone.

while two patients underwent splenic irradiation (6 Gy) to palliate symptomatic splenomegaly. Only one patient (no. 2) had >20% blasts in her bone marrow at the time of transplantation, and one patient (no. 3) was older than four yr at diagnosis.

Donor choice

Serologic typing for HLA-A and -B antigens, and a low-resolution generic DRB1 oligotyping were available for all donor–recipient pairs. High or middle-resolution DNA typing for all loci in unrelated donor–recipient pairs and mismatched family donor–recipient pairs confirmed the previous serological typing.

The patient–donor characteristics are shown in Table 3. Of the 10 patients who received allogeneic HSCT, two patients received allogeneic BMT from fully matched unrelated donor (n = 2), one patient received allogeneic BMT from an antigen-mismatched unrelated donor (n = 1), one patient received fully matched unrelated cord blood (n = 1), two patients received antigen-mismatched unrelated cord blood (n = 2), three patients received matched bone marrow from their siblings (n = 3), and one received BMT from antigen-mismatched family donor (n = 1).

Preparative regimen and transplantation

All 10 patients were conditioned with busulfan 140 mg/m^2 p.o. in divided doses daily for four days (total dose 560 mg/m^2), fludarabine 30 mg/m^2 once daily i.v. for four days (total dose 120 mg/m^2) and melphalan 90–100 mg/m^2 once daily i.v. for two days or 70 mg/m^2 once daily i.v. for three days (total dose 180–210 mg/m^2). Grafts for BMT were non-T-cell-depleted marrow cells, whereas those for CBT were cord blood stem cells. Basically, GVHD prophylaxis for a matched sibling allograft was MTX alone for patients younger than 10 yr old, and Cs-A was added to short-term MTX for patients older than 10 yr old. But they varied and a single administration of MTX, Cs-A + short-term MTX or tacrolimus + short-term MTX was employed in three patients. Four patients given allograft from an alternative donor received tacrolimus and short-term MTX. The combination of Cs-A and short-term MTX was used in three patients who received unrelated CBT. Detailed characteristics regarding transplantation and its outcome are shown in Table 3. This study was carried out according to the guidelines of the Declaration of Helsinki and according to good clinical practice, after informed consent.

Analysis of chimerism

Engraftment of the donor marrow was assayed using STR analysis or FISH with XY chromosome-specific probes.

Results

Engraftment and GVHD occurrence

At a median of 28 days (range, 13–55 days), eight patients had neutrophil engraftment ($>0.5 \times 10^9$/L) and at a median of 49 days (range, 24–138 days), eight patients had an unsupported platelet count of $>50 \times 10^9$/L. Transplant outcomes are detailed in Table 3. Two patients failed to engraft (nos. 4 and 10), of which one had received 3-antigen mismatched cord blood, while the other had received 2-antigen mismatched bone marrow from his mother. This patient possessed an anti-HLA antibody against HLA-DR9, which was also present in the mother. The patient later received a second successful HSCT from an unrelated donor with mismatched antigen, who did not possess HLA-DR9. Eight patients had 97.6–100% donor cells,

Table 3. Transplantation characteristics and outcome

No.	Conditioning Bu (mg/m^2)	Flu (mg/m^2)	L-PAM (mg/m^2)	Donor-stem cell source	HLA matching (DNA)	Cell dose (×10^8/kg)	GVHD prophylaxis	Engraftment donor cell (%; day)	Acute GVHD	Chronic GVHD	Outcome, months (interval after HSCT)
1	560	120	210	MUD-CB	6/6	0.96	MTX + Cs-A	100% (+45)	I	Absent	Alive (69)
2	560	120	200	MUD-BM	6/6	2.06	MTX + FK	97.6% (+29)	0	Lim	Alive (39)
3	560	120	210	MSD-BM	6/6	3.66	MTX	99.2% (+22)	II	Ext	Alive (34)
4–1	560	120	210	MMUD-CB	3/6	0.80	MTX + Cs-A	No	NA	NA	Relapse (day69)
4–2	TBI (10 Gy) + CY (120 mg/kg)			MMUD-BM	4/6	Unknown	MTX + FK	Yes	I	Absent	Relapse (seven after second BMT), died (30)
5–1	560	120	210	MSD-BM	6/6	4.30	MTX + FK	100% (+29)	I	Absent	Relapse (day120)
5–2	TBI (12 Gy) + CY (120 mg/kg) + VP			MUD-BM	6/6	Unknown	MTX + FK	NA	NA	NA	Died (10), IP
6	560	120	200	MUD-BM	6/6	5.20	MTX + FK	100% (+28)	I	Absent	Alive (30)
7	560	120	180	MMUD-BM	5/6	5.00	MTX + FK	99% (+27)	0	Absent	Alive (29)
8	560	120	210	MSD-BM	6/6	4.20	MTX + Cs-A	100% (+28)	0	Absent	Alive (27)
9	560	120	210	MMUD-CB	5/6	1.00	MTX + Cs-A	100% (+113)	IV	Absent	Alive (27)
10–1	560	120	180	MMFD-BM	4/6	4.73	MTX + FK	No	NA	NA	Relapse (day47)
10–2	TBI (12 Gy) + CY (120 mg/kg)			MMUD-BM	5/6	5.34	MTX + FK	Yes	0	NA	Relapse (two after second BMT), died (8)

Bu, busulfan; Flu, fludarabine; L-PAM, melphalan; TBI, total body irradiation; CY, cyclophosphamide; VP, etoposide; GVHD, graft-vs.-host disease; MUD, matched unrelated donor; MSD, matched sibling donor; MMUD, mismatched unrelated donor; MMFD, mismatched family donor; CB, cord blood; BM, bone marrow; MTX, short-term methotrexate; Cs-A, cyclosporin A; FK, tacrolimus; Lim, limited type; Ext, extensive type; NA, not available; HSCT, hematopoietic stem cell transplantation; IP, interstitial pneumonia.

22–113 days after HSCT. Acute GVHD developed in five of eight evaluable patients, grade I in three patients, grade II in one and grade IV in one. Chronic GVHD was observed in two out of eight evaluable patients surviving beyond 100 days after HSCT, with a limited form in one patient and extensive form in the other.

Toxicity and survival

RRT including moderate mucositis, hepatic veno-occlusive disease, cardiac toxicity (of grade II, according to Bearman's grading system) and hemorrhagic cystitis was observed in one patient each; however, none of these complications were fatal. Relapse occurred in three of 10 patients at 69, 120, and 47 days after HSCT, respectively (nos. 4, 5, and 10). These patients received a second BMT from an unrelated donor using a preparative regimen consisting of TBI (10–12 Gy) and cyclophosphamide (120 mg/kg). One patient (no. 5) died of interstitial pneumonitis at the time of second transplantation whereas two other patients (nos. 4 and 10) relapsed again at seven and two months after the second transplantation, respectively, and eventually died. The remaining seven patients are still alive and are in complete remission after HSCT, with a median observation time of 30 months (range, 27–69).

Discussion

The purpose of this study was to improve the outcome of HSCT in JMML using a fludarabine-containing regimen without using TBI. Fludarabine is a nucleoside analogue that has been successfully employed for the treatment of low-grade lymphoid malignancies (9). However, several investigators have reported that it has also been active in cases with acute myeloid leukemia and myelodysplastic syndrome (10). The combination chemotherapy of FLAG (G-CSF) seemed to produce good results in children with relapsed, poor-prognosis acute monocytic leukemia (11). The use of fludarabine may be effective in suppressing the aggressive growth of malignant clone of monocytes in JMML. The second point, which favors the use of fludarabine, is its strong cytotoxic activity against lymphocytes, which consistently prolongs immunosuppression, facilitating the engraftment of hematopoietic stem cells both from HLA-identical siblings and unrelated donors (12). But graft failure was seen in two who had received HLA-mismatched HSCT. To overcome graft failure, particularly in mismatched transplant, it may be necessary to use low-dose TBI or more immunosuppressive agents. Conventional CBT utilized a TBI or a busulfan-based myeloablative conditioning regimen, which carries a high risk of morbidity and mortality (13). On the other hand, Bradley et al. (14) reported that reduced intensity CBT may result in graft failure in specific high-risk chemo-naive patients (chronic myelogenous leukemia, hemophagocytic lymphohistiocytosis, and myelodysplastic syndrome). In our study, two of three patients were successfully transplanted with unrelated umbilical cord blood cells using fludarabine-containing regimen. Although there are very few data in the literature reporting specific results and prognostic factors of CBT in JMML, our experience suggests that a conditioning with fludarabine, busulfan, and melphalan may possibly decrease the mortality rate and the risk of graft failure even in the case of CBT.

Fludarabine in combination with melphalan, cyclophosphamide, or other agents can replace TBI or can be used together with low-dose TBI regimens (15, 16). Occurrence of long-term complications such as growth retardation, infertility (17) and appearance of a second malignancy are the major concerns following TBI therapy in children (18). Therefore, we decided to avoid radiotherapy for treatment of JMML, a condition that occurs during early childhood. In our study, no patient experienced life-threatening regimen-related grade III/IV toxicities, such as severe viral infection, idiopathic pneumonitis, thrombotic microangiopathy, and veno-occlusive disease of the liver. Grade IV acute GVHD developed in one patient (no. 9). In this case, her hepatosplenomegaly progressed, and Cs-A was discontinued on day +17. Following resumed Cs-A therapy, her acute GVHD improved and she has maintained CR. Thus, the preparative regimen consisting of busulfan, fludarabine, and melphalan seems safe, because no patient died of transplantation-related causes. Further long-term follow-up is necessary to evaluate growth retardation, infertility, and second malignancy.

Koyama et al. (19) presented a case using a reduced intensity regimen consisting of fludarabine (30 mg/m^2 for four days) and melphalan (70 mg/m^2 for two days) after AML-type chemotherapy. JMML patients who respond to chemotherapy might be considered as candidates for a non-myeloablative preparative, reduced intensity preparative regimen. Disease recurrence remains the major cause of treatment failure for JMML, and it is believed that both intensive myeloablative conditioning and a graft-vs.-leukemia effect are needed to eradicate the disease (2, 3). Thus, further studies in the future are necessary to compare the results between transplants

conditioned with myeloablative regimens and those conditioned with reduced intensity, non-myeloablative regimens.

A high relapse rate has been the major cause of failure of HSCT in JMML. In patients with JMML, relapse occurs early, generally within the first year after the allograft (20). In this study, only two out of 10 patients bear high-risk features (age more than four yr, blast count at HSCT > 20%) as defined by the EWOG-MDS/EBMT study. The development of chronic GVHD might be associated with better survival, although the association was not significant, possibly because of the small sample size of the study. Seven of 10 patients survived in complete remission for more than two yr after HSCT even though only two patients developed chronic GVHD. Among three patients who relapsed, two failed to engraft, and one had no signs of chronic GVHD. Despite the use of a TBI conditioning regimen for second BMT, two patients without chronic GVHD relapsed again within the first year after BMT. Yoshimi et al. (21) reported that none of the six patients who developed chronic GVHD after second HSCT relapsed. It is reasonable to speculate that chronic GVHD led to a stronger graft-vs.-leukemia effect and resulted in a favorable outcome for allogeneic HSCT in JMML.

Although the number of patients was too small for statistical analysis, this study indicates that a preparative regimen consisting of fludarabine, busulfan, and melphalan can be used satisfactorily in conditioning patients with JMML who receive transplantation, either in form of cord blood or bone marrow not only from HLA-matched siblings but also from alternative donors. Further large studies are needed to confirm any advantage of this choice.

References

1. Niemeyer CM, Aricò M, Basso G, et al. Chronic myelomonocytic leukemia in childhood: A report of 110 cases. Blood 1997: 89: 3534–3543.
2. Manabe A, Okamura J, Yumura-Yagi K, et al. Allogeneic hematopoietic stem cell transplantation for 27 children with juvenile myelomonocytic leukemia diagnosed based on the criteria of the International JMML Working Group. Leukemia 2002: 16: 645–649.
3. Smith FO, King R, Nelson G, et al. Unrelated donor bone marrow transplantation for children with juvenile myelomonocytic leukemia. Br J Haematol 2002: 116: 716–724.
4. Locatelli F, Nöllke P, Zecca M, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): Results of the EWG-MDS/EBMT trial. Blood 2005: 105: 410–419.
5. Matthes-Martin S, Mann G, Peters C, et al. Allogeneic bone marrow transplantation for juvenile myelomonocytic leukemia: A single center experience and review of the literature. Bone Marrow Transplant 2000: 26: 377–382.
6. Korthof ET, Snijder PP, De Graaff AA, et al. Allogeneic bone marrow transplantation for juvenile myelomonocytic leukemia: A single center experience of 23 patients. Bone Marrow Transplant 2005: 35: 455–461.
7. Niemeyer CM, Fenu S, Hasle H, Mann G, Stary J, Van Wering E. Differentiating juvenile myelomonocytic leukemia from infectious disease. Blood 1998: 91: 365–367.
8. Hasle H, Niemeyer CM, Chessells JM, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative disease. Leukemia 2003: 17: 277–282.
9. Keating M, O'Brien S, Lerner S, et al. Long-term follow-up of patients with chronic lymphocytic leukemia (CLL) receiving fludarabine regimens as initial therapy. Blood 1998: 92: 1165–1171.
10. Virchis A, Koh M, Rankin P, et al. Fludarabine, cytosine arabinoside, granulocyte-colony stimulating factor with or without idarubicin in the treatment of high risk acute leukemia and myelodysplastic syndrome. Br J Haematol 2004: 124: 26–32.
11. Fleischhack G, Hasan C, Graf N, Mann G, Bode U. Ida-FLAG (idarubicin, fludarabine, cytarabine, G-CSF) an effective remission-induction therapy for poor-prognosis AML of childhood prior to allogeneic or autologus bone marrow transplantation: Experiences of a phase II trial. Br J Haematol 1998: 102: 647–655.
12. Giralt S, Thall PF, Khouri I, et al. Melphalan and purine analog-containing preparative regimens: Reduced-intensity conditioning for patients with hematologic malignancies undergoing allogeneic progenitor cell transplantation. Blood 2001: 97: 631–637.
13. Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. N Engl J Med 1997: 337: 373–381.
14. Bradley MB, Satwani P, Baldinger L, et al. Reduced intensity allogeneic umbilical cord blood transplantation in children and adolescent recipients with malignant and non-malignant diseases. Bone Marrow Transplant 2007: 40: 621–631.
15. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. Blood 1998: 91: 756–763.
16. Niederwieser D, Maris M, Shizuru JA, et al. Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and post grafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological disease. Blood 2003: 101: 1620–1629.
17. Georgiani G, Bozzola M, Locatelli F, et al. Role of busulfan and total body irradiation on growth of pre-pubertal children receiving bone marrow transplantation and results of treatment with recombinant human growth hormone. Blood 1995: 86: 825–831.
18. Socie G, Curtis RE, Deeg HJ, et al. New malignant diseases after allogeneic marrow transplantation for childhood acute leukemia. J Clin Oncol 2000: 18: 348–357.
19. Koyama M, Nakano T, Takeshita Y, et al. Successful treatment of JMML with related bone marrow transplantation after reduced-intensity conditioning. Bone Marrow Transplant 2005: 36: 453–454.

20. LOCATELLI F, NIEMEYER C, ANGELUCCI E, et al. Allogeneic bone marrow transplantation for chronic myelomonocytic leukemia in childhood: A report from the European Working Group on Myelodysplastic Syndrome in Childhood. J Clin Oncol 1997: 15: 566–573.

21. YOSHIMI A, MOHAMED M, BIERINGS M, et al. Second allogeneic hematopoietic stem cell transplantation (HSCT) results in outcome similar to that of first HSCT for patients with juvenile myelomonocytic leukemia. Leukemia 2007: 21: 556–560.

Disease-associated *CIAS1* mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients

Megumu Saito,[1] Ryuta Nishikomori,[1] Naotomo Kambe,[2,3] Akihiro Fujisawa,[2] Hideaki Tanizaki,[2] Kyoko Takeichi,[4] Tomoyuki Imagawa,[5] Tomoko Iehara,[6] Hidetoshi Takada,[7] Tadashi Matsubayashi,[8] Hiroshi Tanaka,[9] Hisashi Kawashima,[10] Kiyoshi Kawakami,[11] Shinji Kagami,[12] Ikuo Okafuji,[1] Takakazu Yoshioka,[1] Souichi Adachi,[1] Toshio Heike,[1] Yoshiki Miyachi,[2] and Tatsutoshi Nakahata[1]

Departments of [1]Pediatrics and [2]Dermatology, Kyoto University Graduate School of Medicine, Kyoto; [3]Department of Dermatology, Chiba University Graduate School of Medicine, Chiba; [4]Department of Pediatrics, Ehime Prefectural Central Hospital, Matsuyama; [5]Department of Pediatrics, Yokohama City University School of Medicine, Yokohama; [6]Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto; [7]Department of Pediatrics, Kyushu University Graduate School of Medicine, Fukuoka; [8]Department of Pediatrics, Seirei Hamamatsu Hospital, Hamamatsu; [9]Department of Pediatrics, School of Medicine, Hirosaki University, Hirosaki; [10]Department of Pediatrics, Tokyo Medical University, Tokyo; [11]Department of Pediatrics, Kagoshima City Hospital, Kagoshima; and [12]Department of Dermatology, University of Tokyo, Tokyo, Japan

Cryopyrin-associated periodic syndrome (CAPS) is a spectrum of systemic autoinflammatory disorders in which the majority of patients have mutations in the *cold-induced autoinflammatory syndrome (CIAS)1* gene. Despite having indistinguishable clinical features, some patients lack *CIAS1* mutations by conventional nucleotide sequencing. We recently reported a CAPS patient with mosaicism of mutant CIAS1, and raised the possibility that *CIAS1* mutations were overlooked in "mutation-negative" patients, due to a low frequency of mosaicism. To determine whether there were latent mutant cells in "mutation-negative" patients, we sought to identify mutation-associated biologic phenotypes of patients' monocytes. We found that lipopolysaccharide selectively induced necrosis-like cell death in monocytes bearing *CIAS1* mutations. Monocyte death correlated with *CIAS1* up-regulation, was dependent on cathepsin B, and was independent of caspase-1. Cell death was intrinsic to *CIAS1*-mutated monocytes, was not mediated by the inflammatory milieu, and was independent of disease severity or anti–IL-1 therapy. By collecting dying monocytes after lipopolysaccharide treatment, we succeeded in enriching *CIAS1*-mutant monocytes and identifying low-level *CIAS1*-mosaicism in 3 of 4 "mutation-negative" CAPS patients. Our findings reveal a novel effect of *CIAS1* mutations in promoting necrosis-like cell death, and demonstrate that *CIAS1* mosaicism plays an important role in mutation-negative CAPS patients. (Blood. 2008;111: 2132-2141)

Introduction

Cryopyrin-associated periodic syndrome (CAPS) is a spectrum of hereditary periodic fever disorders, and is associated with mutations in the *cold-induced autoinflammatory syndrome (CIAS)1* gene and its encoded protein, cryopyrin.[1] CAPS consists of 3 phenotypically overlapping but relatively distinct syndromes: familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurologic cutaneous and articular (CINCA) syndrome, also known as neonatal-onset multi-inflammatory disease. These syndromes are diagnosed mainly based upon clinical manifestations, among which an urticaria-like rash beginning in the neonatal or early infantile period is common. While FCAS and MWS are characterized by periodic attacks of urticarial rash, fever, and arthralgia, patients with CINCA syndrome, the most severe condition of CAPS, exhibit continuous disease activity, with fever, urticarial rash, arthropathy, chronic meningitis, papilledema, growth and mental retardation, and hearing loss.[1,2]

Recent genetic studies revealed that CAPS patients usually carry heterozygous mutations in the *CIAS1* coding region (mutation-positive patients[3-8]). Although they exhibit no recognizable differences in clinical symptoms or in their response to treatment, approximately half of CINCA syndrome patients lack detectable mutations in *CIAS1*, as assessed by conventional genomic sequencing (mutation-negative patients,[3-5,9-11]), indicating the existence of genetic heterogeneity among CAPS patients. Recently, we reported a patient with CINCA syndrome exhibiting mosaicism of a disease-associated mutation of *CIAS1*[12]. This case suggested that some mutation-negative CAPS patients might have mosaicism of the *CIAS1* mutation; however, the contribution of *CIAS1* mosaicism to disease is controversial. Aksentijevich et al claimed that *CIAS1* mosaicism is a rare event in mutation-negative patients, based on their analysis of 14 patients in which *CIAS1* mosaicism was not identified, even with careful bidirectional sequencing.[11,13,14]

Somatic mosaicism has been reported in a number of autosomal dominant monogenic disorders.[15-17] Diagnosis of mosaicism by conventional genomic sequencing using the dideoxy termination method is often difficult, because the overlapping chromatogram of the mutant is easily missed when the frequency of a mutant allele is less than 20% to 30%.[18] Heteroduplex-based methods[15,19] or subcloning-based analysis of mutant alleles enable one to detect

Submitted June 6, 2007; accepted November 29, 2007. Prepublished online as *Blood* First Edition paper, December 6, 2007; DOI 10.1182/blood-2007-06-094201.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Clinical profiles and *CIAS1* mutations identified in 11 patients with cryopyrin-associated periodic syndrome

Patient number	Diagnosis	Age, y*	Sex	Initial classification	Site of mutation	Biologics therapy	Central nervous system		Skin	Articular		Reference number
							Mental retardation	Meningitis	Urticarial rash†	Arthritis	Contracture	
1	CINCA	2	Female	Mutation-positive	N477K (1431C>A)	None	−	+	+	−	−	(28)
2	CINCA	3	Female	Mutation-positive	G755R (2263G>C)	Anakinra	+	+	+	+	−	(29)
3	CINCA	12	Male	Mutation-positive	M662T (1985T>C)	None	−	+	+	+	−	—
4‡	CINCA	12	Male	Mutation-positive	R260W (778C>T)	None	−	+	+	+	−	—
5	CINCA	13	Male	Mutation-positive	D303N (907G>A)	None	−	+	+	+	−	—
6	CINCA	14	Male	Mutation-positive	Y441H (1321C>T)	Tocilizumab	+	+	+	+	−	—
7	CINCA	15	Male	Mosaic	Y570C (1709A>G)	Anakinra§	−	+	+	+	+	(12)
8	CINCA	18	Female	Mutation-negative	L264F (790C>T)	None	−	+	+	−	−	(30)
9	CINCA	11	Male	Mutation-negative	G307S (919G>A)	None	−	−	+	+	+	—
10	MWS	27	Female	Mutation-negative	E567K (1699G>A)	None	−	Not assessed	+	+	−	(31)
11	CINCA	11	Male	Mutation-negative	Unknown	None	+	+	+	+	−	(32)

+ indicates present; −, not present; and —, not available

*Age at examination.

†Observed since neonatal period.

‡Father has the same heterozygous mutation of *CIAS1*.

§Administration of anakinra began during the study period.

such low-level mosaicism; however, these methods are resource-intensive, and cannot distinguish whether the detected mutation is disease-causing or simply a nonfunctional single nucleotide polymorphism (SNP). An alternative approach involves the isolation of mutant cells using functional analyses based on their characteristic biologic features, and then determining the DNA sequence of the isolated cells. In the current study, we set out to identify specific biologic features of *CIAS1*-mutant cells compared with nonmutated cells, in an effort to specifically isolate *CIAS1*-mutated cells from mutation-negative patients.

CIAS1 is expressed in peripheral blood polymorphonuclear cells (PBMCs), activated T cells, chondrocytes, and most prominently in monocytes.[4] The encoded protein, cryopyrin, belongs to a family of intracellular pattern recognition receptors, which are crucial in the control of immune responses, NF-κB activation, and cell death (reviewed in Ting and Davis[20] and Inohara et al[21]). Cryopyrin associates with caspase-1 and an adaptor protein called apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD: ASC), forming a large protein complex called the inflammasome.[22-24] The inflammasome converts biologically inactive prointerleukin (proIL)-1β to active IL-1β, a potent proinflammatory cytokine, thereby causing an inflammatory response. It is speculated that mutant cryopyrin in CAPS patients activates the inflammasome constitutively in a ligand-independent manner. Indeed, enhanced production of IL-1β has been described in CAPS patients,[3,10,12,25] and is regarded as a primary cause of their inflammatory symptoms. Disease-associated *CIAS1* mutations induce ASC-dependent NF-κB activation in some systems,[12,22,26] and we recently reported that they also induce necrotic cell death in the human monocytic cell line THP-1, which is a novel function of *CIAS1*.[27]

In this study, we explored whether *CIAS1*-mutant cells have specific biologic features, using monocytes from mutation-positive patients, and found that *CIAS1*-mutant monocytes rapidly underwent necrosis-like cell death after treatment with lipopolysaccharide (LPS) to induce *CIAS1* expression. This unique phenotype of *CIAS1* mutant cells enabled us to differentiate *CIAS1*-mutated cells and nonmutated cells in 3 of 4 mutation-negative CAPS patients, and we were able to successfully demonstrate that these 3 patients had mutations of CIAS1 as latent mosaicism.

Methods

Patients

We recruited 11 Japanese CAPS patients, among whom 10 patients had clinically diagnosed CINCA syndrome, and 1 was diagnosed with MWS. All of the CINCA patients met previously described diagnostic criteria.[5,10] The MWS patient had recurrent episodes of inflammation that were not triggered by cold, progressive deafness, and amyloidosis. Written informed consents were obtained from the patients and their families, according to the protocol of the institutional review board of Kyoto University Hospital and in accordance with the Declaration of Helsinki. Details of patients are described in Table 1.

Reagents

Crude LPS (cLPS) from *Escherichia coli* O127:B8, muramyl dipeptide (MDP) and actinomycin D were purchased from Sigma-Aldrich (St Louis, MO). Pure LPS (pLPS), Pam3CSK, Poly I:C, recombinant flagellin, single-strand RNA (ss-RNA) and CpG DNA (type C, ODN M362) were from InvivoGen (San Diego, CA). Z-Tyr-Val-Ala-Asp(OMe)-CH_2F (YVAD-fmk), [L-3-*trans*-(propylcarbamoyl) oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA074-Me), and MG-132 were obtained from EMD (Darmstadt, Germany).

Culture of primary human cells

PBMCs were obtained from CAPS patients and healthy volunteers. $CD14^+$ monocytes and $CD14^-$ cells were purified by AutoMACS (Miltenyi Biotec, Gladbach, Germany), or sorted using a FACSVantage System (BD, Franklin Lakes, NJ). The purity of $CD14^+$ monocytes purified by AutoMACS and FACSVantage was more than 75% and 95%, respectively. PBMCs were cultured in RPMI1640 containing 10% fetal calf serum at a density of 10^6/mL with cLPS, MDP, or Toll-like receptor (TLR) ligands. In some experiments, PBMCs were preincubated for 30 minutes with YVAD-fmk (50 μM), CA074-Me (50 μM), or MG-132 (5 μM) before cLPS treatment.

Genetic analysis

Genomic DNA from PBMCs or whole blood was obtained as previously described.[12] To analyze the frequency of the *CIAS1*-mutant allele, exon 3 or the entire coding region of *CIAS1* was amplified using high fidelity DNA polymerase KOD plus (Toyobo, Osaka, Japan) and subcloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA).[12] Genomic DNA and subclones were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR

Total RNA was extracted from 1 to 2 × 10^5 monocytes using an RNeasy Mini kit (Qiagen, Venlo, Netherlands). After DNase I digestion (Invitrogen) and first-strand cDNA synthesis with Sensiscript RT (Qiagen), the products were subjected to real-time quantitative polymerase chain reaction (PCR) analysis of *CIAS1* and 18S rRNA (as an internal control) using an ABI PRISM 7900HT (Applied Biosystems). The following primers (200 nM) and probes (100 nM) were used: *CIAS1*, forward 5′-GAGCCTCAACAAACGCTACACA-3′, reverse 5′-CTTGCCGATGGCCAGAAG-3′, probe 5′-FAM-CTGCGTCTCATCAAGGAGCACCGG-TAMRA-3′; 18s rRNA, forward 5′-AGTCCCTGCCCTTTGTACACA-3′, reverse 5′-GATCCGAGGGCCTCACTAAAC-3′, probe 5′-FAM-CGCCCGTCGCTACTACCGATTGG-TAMRA-3′. Expression of *CIAS1* was normalized to 18S rRNA; expression is shown relative to *CIAS1* expression in the absence of LPS stimulation, which was set equal to one.

Allele-specific PCR

The PCR settings are described in Table S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) and were optimized to detect at least 0.6% of mutant alleles for each mutation. To make dilution series, patient DNA was diluted with DNA from an individual who was proved not to have latent mosaicism of *CIAS1* (confirmed by subcloning).

Flow cytometry

PBMCs were incubated with a FITC-conjugated anti–human CD14 mAb (BD), then sorted and analyzed using a FACScalibur System (BD). For intracellular IL-1β staining, cells were fixed, permeabilized, washed with Permfix and Cytoperm (BD), and incubated with a PE-conjugated anti–human IL-1β mAb (BD), at a concentration of 0.5 μg/mL. Cells were also incubated with propidium iodide (PI) or 7-aminoactinomycin D (7-AAD, purchased from BD) to identify nonviable cells. The expression of phosphatidylserine in the external layer of the plasma membrane was evaluated using PE-conjugated annexin-V (BD). In mutation-negative patients (patients 7-11), LPS-stimulated $CD14^+/PI^+$ cells and $CD14^+/PI^-$ cells were sorted using the FACSVantage System.

Cell viability assay

After incubation with or without cLPS, purified monocytes were centrifuged onto glass slides and were subjected to Giemsa staining. Cell viability was determined using a trypan blue exclusion assay.

Plasmids and cell lines

Expression plasmids for *CIAS1* and ASC in the pEF-BOS vector background have been described previously.[12] *CIAS1* mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).[12] The ability of each construct to induce NF-κB activation was assessed using a dual luciferase reporter assay in HEK293 cells as previously described.[12] cDNAs encoding carboxy-terminal green fluorescent protein (GFP)-tagged *CIAS1* and GFP-tagged mutants of *CIAS1* were subcloned into pcDNA/5TO (Invitrogen), before being introduced into human monocytic THP-1 cells using the Cell Line Nucleofector Kit V (Amaxa Biosystems, Cologne, Germany). Phorbol myristate acetate (10 ng/mL) was added to improve gene expression and reduce spontaneous cell death.[27] Four hours after the introduction of plasmids, cell death in GFP-positive THP-1 cells was measured by flow cytometry.

Statistical analysis

Data are represented as means plus or minus SD. Statistical analysis was performed using the Students *t* test. *P* less than .05 was considered to be statistically significant.

Results

Patient profile

To explore whether mutation-negative CAPS patients were misdiagnosed due to *CIAS1* mosaicism and the low frequency of the mutant allele, we recruited 11 Japanese CAPS patients. Clinical and genetic information for these patients is described in Table 1. Among them, 6 CINCA syndrome patients (patients 1-6) were confirmed as heterozygous for *CIAS1* mutation by ordinary genomic sequencing, and classified as mutation-positive patients, showing a frequency of the mutant allele of approximately 50% (data not shown). Patient 7 was previously diagnosed as a mosaic, in whom the frequency of the *CIAS1* mutant allele in whole blood was approximately 12%, reflecting that approximately 24% of blood cells carried the *CIAS1* mutation.[12] Three CINCA syndrome patients (patients 8-10) and 1 MWS patient (patient 11) had no evidence of overlapping peaks in their sequencing histograms, indicating that there were no point mutations in *CIAS1* that would correlate with amino acid substitutions, based on repeated bidirectional genomic sequencing. These patients were categorized as mutation-negative patients.

Spontaneous IL-1β production in monocytes at single-cell level

To find possible latent mosaicism in mutation-negative patients, we sought a method which enables mutant and normal cells from the reported mosaic patient to be distinguished at the single cell level. We investigated intracellular IL-1β staining as a candidate technique, because it is generally believed that enhanced production of IL-1β is the underlying molecular cause of CAPS.[10,33-35] To verify this, we first explored in vitro intracellular IL-1β production from CD14-positive peripheral monocytes by flow cytometry. Constitutive IL-1β up-regulation in monocytes was not detected in healthy controls (n = 10), but was observed in the confirmed mosaic patient (patient 7) before starting anakinra treatment (Figure 1). However, we were unable to resolve distinct high-producing ($IL\text{-}1\beta^{high}$) and low-producing ($IL\text{-}1\beta^{low}$) subpopulations of CD14-positive monocytes. In addition, while we sorted out relatively higher- and lower-intensity intracellular IL-1β fluorescence-staining populations, the frequency of the *CIAS1* mutant allele in each population was almost comparable (data not shown). Furthermore, when patient 7 was treated with anakinra, spontaneous IL-1β

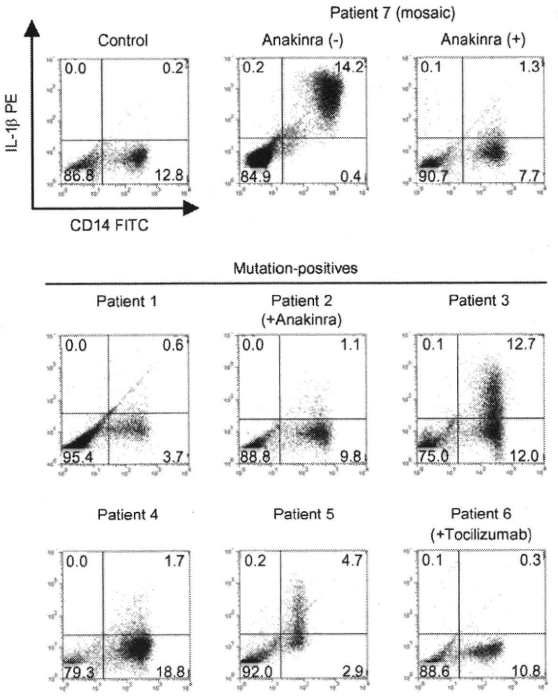

Figure 1. Intracellular IL-1β levels in PBMCs from CAPS patients. PBMCs from healthy controls (n = 10), mutation-positive patients (n = 6), or a mosaic patient (patient 7) were isolated and cultured in vitro for 24 hours. Flow cytometric analysis of CD14 expression and intracellular IL-1β levels was performed. The ratio of $CD14^{+}IL\text{-}1\beta^{high}$ monocytes (cells in the right upper quadrant) in healthy controls was 0.26% (± 0.43%; mean ± SD). Data from each of the indicated patients and a representative healthy control are shown. Numbers in each quadrant are the percentages of total cells.

up-regulation was canceled; therefore, the status of IL-1β production did not distinguish between monocytes with *CIAS1* mutations and those without. Two mutation-positive patients (patient 3 and 5) showed spontaneous up-regulation of intracellular IL-1β in monocytes upon culture of their PBMCs in vitro (Figure 1); this correlated with elevated IL-1β levels in culture supernatant (data not shown). The remaining mutation-positive patients, particularly those who received therapeutic biologics such as anakinra or the anti–IL-6R antibody tocilizumab (patients 2 and 6, respectively), did not show evidence of up-regulation of intracellular IL-1β. The status of intracellular IL-1β therefore seemed to be affected by various factors, such as the treatment applied. Two of the 4 mutation-negative patients (patients 10 and 11, data not shown) also exhibited an up-regulation of intracellular IL-1β. These results show that alterations in intracellular IL-1β levels were not helpful in finding latent mosaicism in mutation-negative patients.

Monocytes from CAPS patients rapidly undergo necrotic cell death when treated with LPS

We recently reported that overexpression of disease-associated mutants of *CIAS1* caused rapid necrosis-like cell death in a human monocytic cell line, THP-1.[27] As *CIAS1* is regulated by NF-κB, which is up-regulated by treatment with LPS,[36] we next verified whether LPS-induced up-regulation of CIAS1 could induce cell death in *CIAS1*-mutated monocytes. Quantitative mRNA analysis of *CIAS1* in mutation-positive patients and in healthy controls revealed that both cLPS and pLPS markedly enhanced *CIAS1* mRNA expression (Figure 2A). When stimulated with cLPS, the number of monocytes from all of the CAPS patients carrying a heterozygous *CIAS1* mutation (patients 1-6) decreased significantly, while in healthy controls, it did not (n = 10; Figure 2B,C). Although the magnitude of the decrease in monocyte number varied for each patient, this phenomenon was observed consistently, regardless of the disease severity, the level of spontaneous IL-1β production, or the therapeutic treatment regimen. Monocyte cell number was reduced to equal levels before and after anakinra treatment of patient 4, even though the clinical response to anakinra was excellent (data not shown). These results suggested that monocytes with *CIAS1* mutations undergo cell death when *CIAS1*

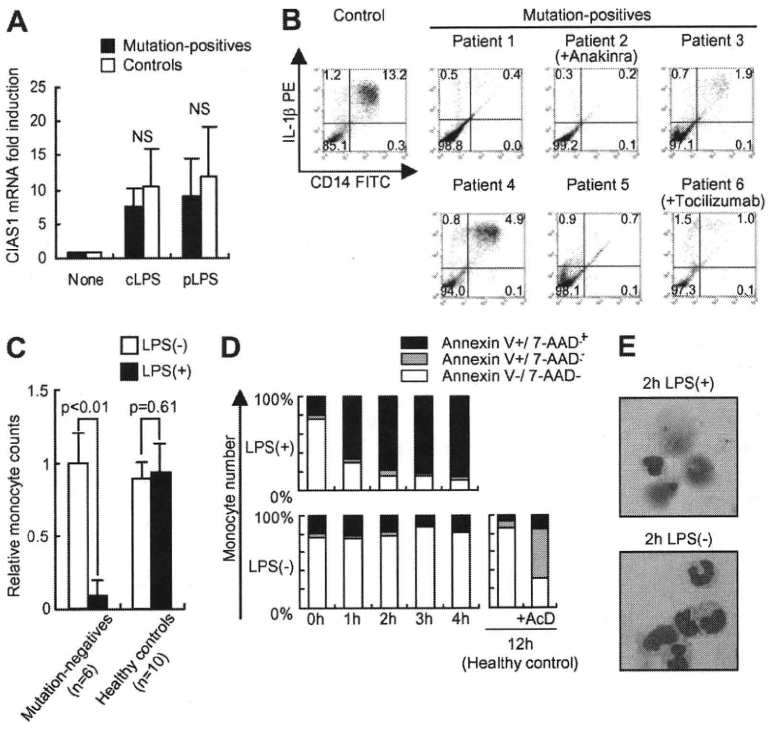

Figure 2. Monocytes from mutation-positive patients rapidly undergo cell death when treated with LPS. (A) Real-time quantitative reverse transcription (RT)-PCR analysis of *CIAS1* mRNA. Purified monocytes were incubated with cLPS (10 ng/mL) or pLPS (10 ng/mL) for 1 hour, and were then subjected to quantitative real-time RT-PCR analysis. Data were normalized to 18S rRNA expression, and represent the means (± SD) of 4 mutation-positive patients or 7 healthy controls. NS: statistically not significant. (B) Intracellular IL-1β staining of PBMCs from CAPS patients. PBMCs from mutation-positive patients (n = 6) or healthy controls (n = 10) were incubated with cLPS (10 ng/mL) for 24 hours. Flow cytometric analysis was performed as described for Figure 1. Representative results of each patient, or the control healthy patients, are shown. Numbers in each quadrant are the percentages of total cells. (C) Monocyte cell number decreases upon LPS stimulation. PBMCs were incubated with or without cLPS (10 ng/mL) for 24 hours, as described in Figure 1. Data represent the number of CD14-positive cells in 10 000 PBMCs, compared with the preincubation state, and represent the means (± SD) of the indicated number of patients. (D) PBMCs from mutation-positive patients were stained with FITC-conjugated anti-CD14, PE-conjugated anti–annexin V, and 7-AAD. Samples were analyzed by flow cytometry, gated to select CD14-positive cells, and then analyzed for cell viability. Data from normal PBMCs that were treated with actinomycin D (AcD, 1 μg/mL) for 12 hours were used as a positive control for apoptotis. Representative data from 5 mutation-positive patients are shown. (E) Giemsa staining of monocytes from mutation-positive patients incubated for 2 hours with or without cLPS. Cells were observed by inverted microscopy using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a 40×/0.85 NA objective lens, an Olympus DP70 camera, and DP-controller version 1.1 software (Olympus). Data are representative of 3 mutation-positive patients.

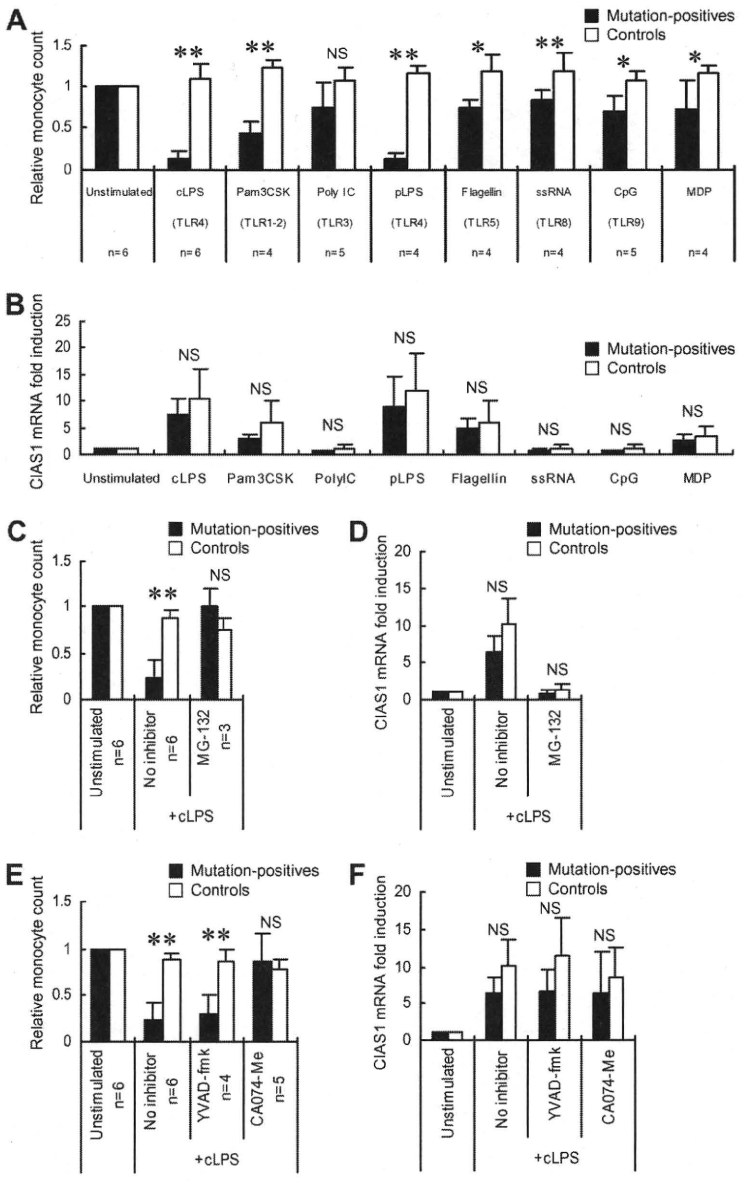

Figure 3. Effects of TLR ligands and various inhibitors on monocyte cell death. (A) PBMCs from mutation-positive patients or healthy controls were incubated for 24 hours with cLPS (10 ng/mL), MDP (5 μg/mL), Pam3CSK (10 μg/mL), Poly I:C (25 μg/mL), pLPS (10 ng/mL), recombinant flagellin (100 ng/mL), single-stranded RNA (ssRNA, 2 μg/mL), or CpG DNA (1 μM). The number of $CD14^+$ cells in 10 000 PBMCs was determined; the ratio of $CD14^+$ cells in stimulated PBMCs to those in unstimulated PBMCs is presented. Data represent the means (± SD) of the indicated number of patients or healthy controls (n = 5). (B) Real-time quantitative RT-PCR analysis of *CIAS1* mRNA. Purified monocytes were stimulated with TLR ligands or MDP, as described for (A), for 1 hour, and were then subjected to quantitative real-time RT-PCR analysis. Data were normalized to 18S rRNA expression, and represent the means (± SD) of 4 mutation-positive patients or 7 healthy controls. (C, E) PBMCs from the indicated number of mutation-positive patients or healthy controls (n = 4) were incubated with or without MG-132 (5 μM), YVAD-fmk (50 μM), or CA074-Me (50 μM) for 30 minutes before the addition of cLPS. The cells were then incubated with or without cLPS (10 ng/mL) for 4 hours. The ratio of $CD14^+$ and 7-AAD^- cells in 10 000 PBMCs relative to untreated cells is shown; data represent the means (± SD) (D, F) Real-time quantitative RT-PCR analysis of *CIAS1* mRNA. Purified monocytes were preincubated with the indicated inhibitors for 30 minutes, as described for (C and E), and were then stimulated with cLPS (10 ng/mL) for 1 hour. Thereafter, cells were collected and subjected to quantitative real-time RT-PCR analysis. Data were normalized to 18S rRNA expression. Values represent the means (± SD) of 4 mutation-positive patients or 7 healthy controls. **P* less than .05, ***P* less than .01, NS: not significant, compared with healthy controls by Student *t* test.

expression is induced by LPS. To confirm these results, flow cytometric analysis with PI or 7-AAD staining was performed. As shown in Figure 2D, CD14-positive monocytes from all of the heterozygous CAPS patients rapidly underwent cell death when treated with cLPS, while CD14-negative PBMCs did not (data not shown). When annexin-V staining was also examined, we found no evidence of a population of early apoptotic cells, defined as being annexin-V^+/7-AAD^- (lower right graph in Figure 2D); rather, cells became annexin-V/7-AAD double-positive directly, implying that they underwent necrosis, not typical apoptosis (Figure 2D). This result was comparable with previous observations using THP-1 cells.[27] Giemsa staining of cLPS-stimulated purified monocytes showed evidence of cellular debris from disrupted cells, but failed to detect typical apoptotic morphologic features, such as nuclear condensation or apoptotic bodies, even at early time points (1 or 2 hours) after cLPS stimulation (Figure 2E). Both cLPS and pLPS were equally effective in inducing monocyte death, excluding the possibility that unknown materials in cLPS were responsible for the effect (see Figure 3A). Thus, monocytes isolated from CAPS patients with *CIAS1* mutations rapidly underwent a type of cell death consistent with necrosis, rather than apoptosis, when treated with LPS.

Effect of TLR ligands on CIAS1 mutant monocytes

LPS is a ligand of TLR4,[37-39] which is constitutively expressed on monocytes.[40] Because monocytes express several types of TLR,[40] we investigated whether other TLR ligands were also able to induce cell death in *CIAS1*-mutant monocytes. As shown in Figure 3A, while several TLR ligands mildly or moderately decreased the number of monocytes from mutation-positive patients, none of them were as potent as LPS. MDP, a bacterial compound that was previously reported to be an activator of the cryopyrin inflammasome,[41] also mildly decreased monocyte number. Because some TLRs, such as TLR3 (receptor for poly I:C) and TLR9 (receptor for CpG DNA) are not typically expressed on monocytes,[40] modification of the cytokine milieu by other peripheral blood cells may have partly affected the fate of the mutant monocytes. Quantitative mRNA analysis of *CIAS1* in mutation-positive patients and in the healthy controls revealed that the effects of Pam3CSK and flagellin

on *CIAS1* expression were relatively mild, and the effects of poly I:C, single-stranded RNA, and CpG DNA were negligible (Figure 3B), consistent with the finding that LPS is the strongest inducer of cell death of *CIAS1* mutant monocytes. Furthermore, to inhibit the LPS-induced up-regulation of CIAS1 we pretreated monocytes with MG-132, a broadly effective proteasome inhibitor also known as a NF-κB inhibitor. The effect of LPS on monocyte death (Figure 3C) and *CIAS1* mRNA induction (Figure 3D) was completely abrogated. Although the possibility that MG-132 directly inhibits monocyte death cannot be excluded, these results suggested that the effect of LPS and other TLR ligands in inducing monocyte cell death is associated with their ability to enhance *CIAS1* expression.

LPS-induced death of monocytes with CIAS1 mutations is cathepsin B–dependent

Caspase-1 is a key downstream molecule of cryopyrin in IL-1β processing, and forms part of the inflammasome, along with cryopyrin and ASC.[23,42] We recently reported that the rapid necrosis of THP-1 cells induced by *CIAS1* disease-associated mutants was not mediated by a caspase-1-dependent pathway, but occurred in a lysosomal cathepsin B–dependent manner.[27] To explore the contribution of caspase-1 or cathepsin B in the LPS-induced cell death of *CIAS1*-mutant human primary monocytes, we pretreated PBMCs from mutation-positive patients with a caspase-1 inhibitor, YVAD-fmk, or a cathepsin B-specific inhibitor, CA074-Me (Figure 3E). YVAD-fmk was not effective in preventing monocyte cell death, even at a concentration which substantially suppressed LPS-induced IL-1β production (Figure S1). In contrast, CA074-Me effectively protected mutation-positive monocytes from LPS-induced cell death. Neither YVAD-fmk nor CA074-Me affected the up-regulation of *CIAS1* mRNA by LPS (Figure 3F), indicating that the effect of CA074-Me was not at the level of *CIAS1* expression, but that it inhibited a posttranscriptional pathway mediated by the mutant form of cryopyrin. Taken together, our findings implied a novel function of mutated *CIAS1* in inducing monocyte death in a cathepsin B-dependent, caspase-1–independent manner. The results also suggested that LPS-induced death of monocytes carrying disease-associated mutations of *CIAS1* is mediated by a similar mechanism to that reported for the cell death of human monocytic THP-1 cells induced by overexpression of mutated *CIAS1*.

Selective induction of cell death of CIAS1-mutated monocytes

To confirm that the cell death of *CIAS1* mutant monocytes was due to an intrinsic mechanism, rather than the result of modifications of the inflammatory milieu, we purified monocytes using magnetic sorting, stimulated them with LPS, and performed a trypan blue exclusion assay. We observed rapid cell death of purified monocytes upon cLPS stimulation, indicating that the monocytic death observed in the previous experiments was not mediated by other cells (Figure 4A). Giemsa staining of the purified monocytes also revealed features of necrosis-like cell death, as seen previously (Figure 2E).

Because patient 7 had mutant and nonmutant cells in the same milieu due to confirmed *CIAS1* mosaicism, we were interested in whether LPS-induced cell death was selective for monocytes carrying the *CIAS1* mutation. The percentage of CD14-positive monocytes from patient 7 tended to decrease slightly upon cLPS stimulation, but the cell survival profile was more similar to normal controls (Figure 4B). When the DNA of the monocytes from patient 7 was extracted and sequenced, there was an overlapping, small peak on the sequencing chromatogram in unstimulated PBMCs,

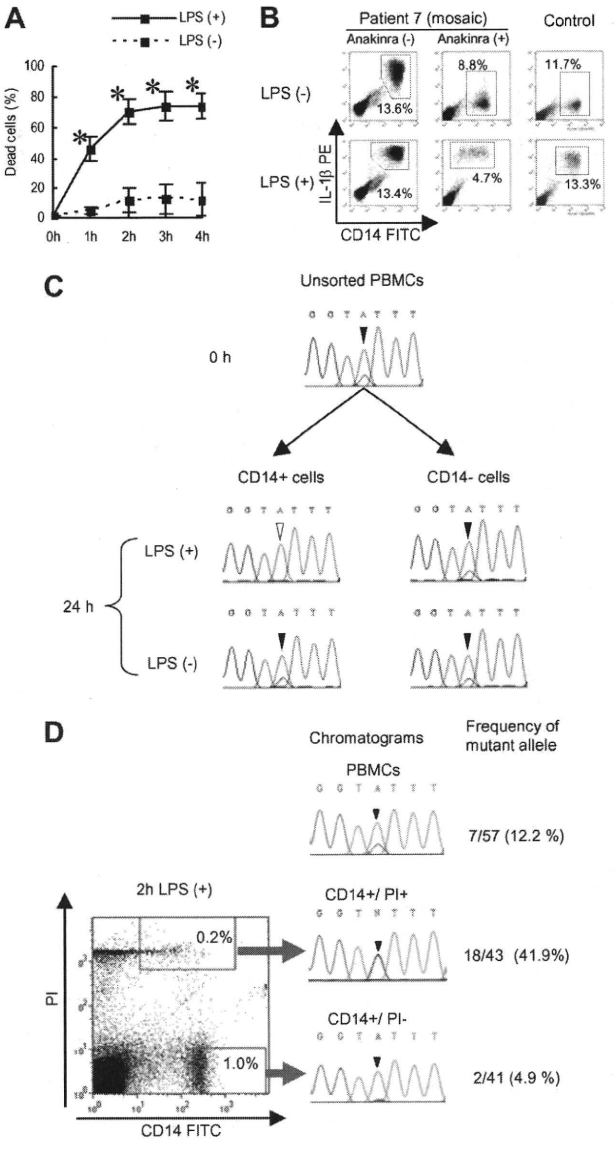

Figure 4. Selective elimination of mutated monocytes from mutation-positive and mosaic patients. (A) Trypan blue exclusion assay of purified monocytes. Purified monocytes were incubated with or without cLPS (10 ng/mL) and dead cells were counted. Values represent the means (± SD) of 3 mutation-positive patients. **P* less than .01 compared with LPS (-) counterparts. (B) Representative intracellular IL-1β staining of PBMCs from patient 7 or healthy controls after incubation with or without cLPS (10 ng/mL) for 24 hours. Numbers in each rectangle are the percentages of total cells. (C) PBMCs from patient 7 were cultured with or without cLPS (10 ng/mL) for 24 hours. CD14-positive and -negative cells were sorted, and DNA was extracted and sequenced for analysis of *CIAS1*. Chromatograms of the *CIAS1* gene at nucleotide position 1709 (black or white arrowhead) from each population of cells are shown. Note that the overlapping "G" peak (black arrowhead) disappeared from LPS-treated CD14-positive cells. The data are representative of 3 independent experiments. (D) *CIAS1*-mutated monocytes from a mosaic patient were enriched in the dying cell population. Left panel: flow cytometry data of PBMCs from patient 7 stimulated with cLPS for 2 hours. Right panel: chromatograms of the *CIAS1* gene at position 1709 (arrowhead), and frequency of the mutant allele as determined by subcloning from each population. The data are representative of 3 independent experiments.

indicating a mutation of the guanine (G) nucleotide at position 1709 (Figure 4C). In monocytes stimulated with cLPS, the histogram peak corresponding to the mutated G disappeared, while in unstimulated monocytes, or cLPS-stimulated, CD14-negative PBMCs, it did not (Figure 4C). These results suggested that cell death was induced selectively in *CIAS1*-mutated monocytes. When

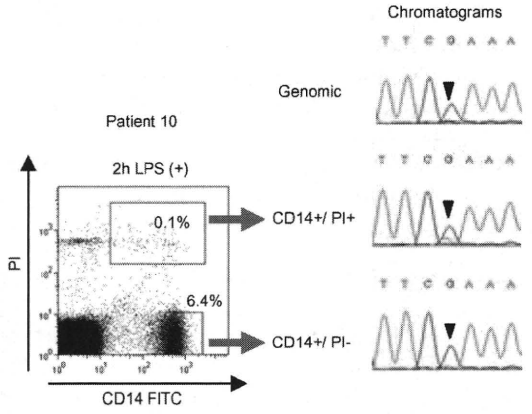

Figure 5. Enrichment of *CIAS1*-mutated monocytes from a *CIAS1* mutation-negative patient. Flow cytometry analysis of PBMCs from patient 10 stimulated with cLPS for 2 hours (left panel), and chromatograms of the *CIAS1* gene at position 1699 from each of the populations of cells (right panel). Numbers in each rectangle are the percentages of total cells.

we sorted CD14-positive/PI-negative cells and CD14-positive/PI-positive cells from cLPS-stimulated PBMCs from patient 7 (Figure 4D) and performed genomic DNA sequencing of each cell population, we found that *CIAS1*-mutated cells were enriched in the PI-positive population, and eliminated from the PI-negative population. Subcloning-based frequency analysis revealed successful enrichment of the mutant G allele, from a frequency of 12.2% in PBMCs to 41.9% in the PI-positive population of dying monocytes, correlating with an enrichment of mutated cells from approximately 25% to more than 80%. The frequency of the mutant allele in PI-negative monocytes that were not undergoing cell death significantly decreased, to 4.9%. These results suggested that cell death was induced exclusively in *CIAS1*-mutated monocytes, and not in normal cells, even though both cell types were exposed to the same extracellular milieu.

Identification of mosaicism in 3 of 4 CIAS1 mutation-negative CINCA patients

Based on the results from patient 7, we set out to identify *CIAS1* mosaicism in the remaining mutation-negative patients by enriching for PI^+, dying monocytes after cLPS stimulation. We stimulated PBMCs from patients 8-11 with cLPS, and while the decrease in monocyte cell number was comparable with normal controls (data not shown), we were able to sort dying (PI^+) from viable (PI^-) monocytes. Subsequent sequencing of the population of monocytes undergoing cell death revealed an overlapping peak on the sequencing chromatogram, indicating mosaicism, in 3 of 4 patients. The nucleotide substitutions were as follows (parentheses indicate the corresponding amino acid change): 790C > T (L264F) in patient 8; 919G > A (G307S) in patient 9; and 1699G > A (E567K) in patient 10 (Figure 5 and Figure S2). Overlapping peaks were not obvious in either chromatogram from unstimulated PBMCs or cLPS-stimulated, PI-negative monocytes. Subcloning analysis of genomic DNA from whole blood revealed that the mutant allele was enriched from 4.3% (2/47) to 19.4% (7/36) in patient 8, 4.3% (2/47) to 11.1% (3/27) in patient 9, and 6.5% (3/46) to 15.2% (7/46) in patient 10 (Table 2). The mutations and corresponding amino acid changes in patients 8, 9, and 10 have not been previously reported as either mutations or SNPs, and were not observed among 100 healthy Japanese donors (data not shown). We confirmed the existence of latent mosaicism in these patients by allele-specific PCR, which can detect mutant alleles at a frequency of 0.6% (Figure 6). We also analyzed 100 healthy controls to exclude the possibility of latent mosaicism among this population, and found no evidence of mutant *CIAS1* alleles (Figure 6 and data not shown). Thus, by selectively inducing cell death with LPS, we successfully diagnosed 3 CAPS patients who had been designated mutation-negative by conventional sequencing. Patient 11 had a phenotype that was marked by severe mental and developmental retardation. When we stimulated PBMCs from patient 11 with cLPS, there was very little cell death of monocytes, and we could not resolve overlapping peaks on the sequencing chromatogram of the population of dying monocytes (data not shown). We generated at least 54 subclones of the entire *CIAS1* coding region from patient 11, and confirmed that this individual did not carry any mutations, even as a mosaicism of *CIAS1*.

Table 2. Frequency of mutant alleles detected in mutation-negative patients

	Patient 8	Patient 9	Patient 10
Site of mutation	790C>T (L264F)	919G>A (G307S)	1699G>A (E567K)
Frequency of mutant allele			
Whole blood	2/47 (4.3%)	2/47 (4.3%)	3/46 (6.5%)
$CD14^+/PI^+$	7/36 (19.4%)	3/27 (11.1%)	7/46 (15.2%)
$CD14^+/PI^-$	2/46 (4.3%)	1/38 (2.6%)	3/48 (6.3%)

Disease-associated mutant CIAS1 induces cell death on THP-1 and spontaneous NF-κB activation

To evaluate whether the newly identified *CIAS1* mutations of this study were relevant to disease manifestation, we examined their ability to rapidly induce necrotic cell death when transiently expressed in human monocytic THP-1 cells (Figure 7A). The mutants identified in the mosaics (patients 7-10, Y570C, L264F,

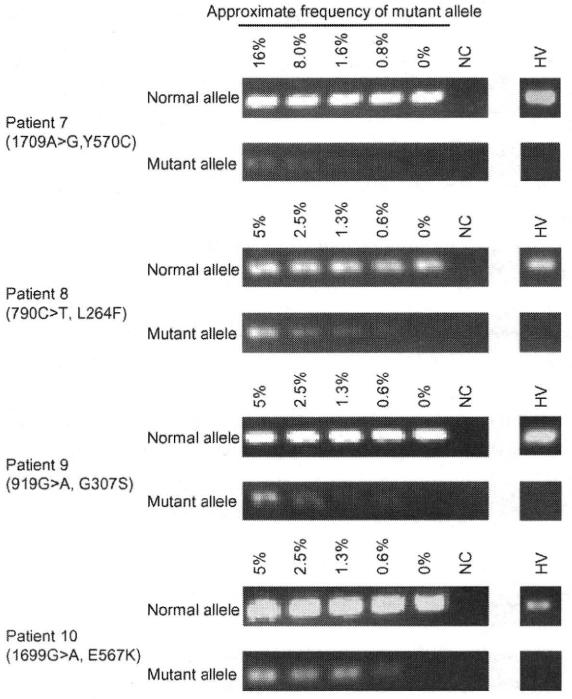

Figure 6. Allele-specific PCR for mutant alleles detected in mosaic patients. PCR was performed with mutant or normal allele-specific primers and the corresponding reverse primer (see Table S1). Dilution series were made by mixing patients' DNA and DNA from an individual who was proved not to have latent mosaicism of CIAS1. Representative results of mosaic patients and 100 healthy volunteers (HV) are shown. NC indicates negative control.

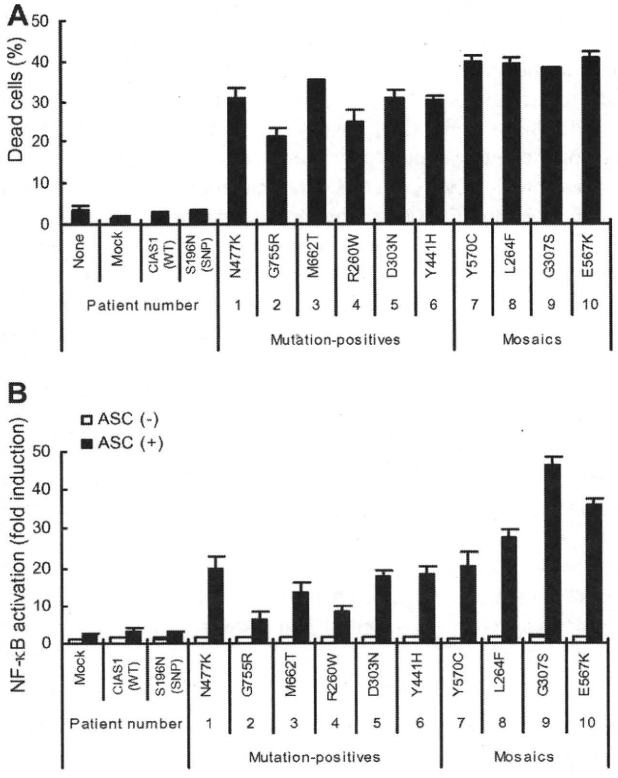

Figure 7. Effect of *CIAS1* mutation on the induction of cell death and ASC-dependent NF-κB activation. (A) 10^6 THP-1 cells were transfected with 0.5 μg of an expression vector for GFP-tagged *CIAS1* wild-type (WT), *CIAS1* SNP S196N, or one of the disease-associated mutants of *CIAS1* (R260W, L264F, D303N, G307S, Y441H, N477K, E567K, Y570C, M662T, and G755R), and incubated with PMA (10 ng/mL) for 4 hours. The percentage of dead cells (7-AAD-positive) among the population of GFP-positive cells is shown. Data represent the means (± SD) of triplicate determinations, and are representative of 3 independent experiments. (B) HEK293FT cells were transfected with 16 ng of an expression vector for *CIAS1*, or one of its mutants, in the presence or absence of 16 ng of an expression vector for ASC. The induction of NF-κB is shown as fold-change compared with cells that were transfected with a control vector without ASC (set equal to one). Values are the means (± SD) of triplicate determinations, and data are representative of 2 independent experiments.

G307S, and E567K) had more potent effects than those of the mutation-positive individuals. Transient coexpression of disease-associated *CIAS1* mutants and ASC in HEK293FT cells also caused enhanced NF-κB reporter activity, compared with the expression of wild-type *CIAS1*, or *CIAS1*-SNP S196N, a disease-unrelated *CIAS1* variant (Figure 7B).[12] Note that NF-κB activation induced by the mutants identified in mosaic patients was higher than that induced by the mutants identified in mutation-positive patients (patient 1-6; Figure 7B). These findings provided additional evidence that mutations of *CIAS1* that are found in both mutation-positive and mosaic patients are functional and related to the development of disease-related symptoms in CAPS patients.

Discussion

In the current study, we demonstrated that monocytes carrying disease-associated *CIAS1* mutations rapidly undergo cell death upon induction of *CIAS1* expression by LPS treatment. Selective induction of monocyte cell death was independent of the disease severity, level of IL-1β production, or therapeutic regimen. Subcloning and analysis of transient expression in cell lines indicated that the ability to induce cell death was specific to disease-associated mutations in *CIAS1*. Consequently, we were able to use this distinct biologic characteristic to enrich for monocytes carrying *CIAS1* mutations. We performed genetic analysis on 4 mutation-negative CAPS patients and found *CIAS1* mosaicism in 3 patients, while in the fourth we were able to exclude the presence of a mutation in the *CIAS1* coding region. These findings suggest that a majority of *CIAS1* mutation-negative patients have disease-associated mutations of *CIAS1* as a latent, low-level mosaicism.

Our results indicated that a small number of monocytes carrying *CIAS1* mutations are sufficient to evoke systemic inflammation in *CIAS1* mosaic patients. The question then arises of how such a small number of mutant cells cause the severe inflammatory responses observed in CAPS patients. As shown in Figure 1, we could not distinguish between *CIAS1*-mutated and nonmutated cells by intracellular IL-1β staining of PBMCs in patient 7 (mosaic patient); both mutated and nonmutated monocytes appeared to have similar levels of IL-1β. In patients who were treated with the IL-1 receptor antagonist anakinra, not only was IL-1β signaling blocked, but IL-1β production in peripheral blood monocytes was also dramatically reduced (Figure 1). This finding indicates that modification of the cytokine milieu due to the production of IL-1β by *CIAS1*-mutated cells, which possess constitutive IL-1β producing activity, may cause up-regulation of IL-1β in nonmutated monocytes, thereby leading to a systemic inflammatory condition. In support of this hypothesis, the addition of a neutralizing anti–IL-1β antibody to cultures of PBMCs from patient 7 reduced the levels of intracellular IL-1β, and addition of exogenous IL-1β to control, nonmutated monocytes induced an up-regulation in intracellular IL-1β (data not shown). In addition, we used transient transfection experiments to demonstrate that all of the *CIAS1* mosaic mutants (Y570C, L264F, G307S, and E567K) have the potential to induce higher NF-κB activity compared with wild-type *CIAS1* (Figure 7); thus, in patients in vivo, these mutations could be highly active, and sufficient to evoke severe systemic inflammation, even when present as a low-level mosaicism.

The clinical symptoms of mosaic patients appear to be milder than those of heterozygous patients. Patient 7 carried a *CIAS1*-Y570C mutation, which is one of the most common *CIAS1* mutations, and is associated with a very severe phenotype including mental retardation and epilepsy.[3,5,43] Patient 7 appeared to have milder symptoms than other reported heterozygous patients carrying the same mutation of *CIAS1*, showing neither mental retardation nor epilepsy, even at 15 years of age. Similarly, patient 8, who had the *CIAS1*-L264F mutation as mosaicism, exhibited a milder phenotype than patients with the heterozygous L264F mutation.[11] Although the *CIAS1*-G307S mutation in patient 9 has not yet been reported, the symptoms of the patient also seemed to be milder than that of a patient reported to have the G307V mutation.[44] The relatively mild phenotypes in mosaic patients, despite the relatively potent effects of their mutations on cellular activity (Figure 7), may be attributable to the lower dose of active mutation. Further study with more CAPS patients with CIAS1 mosaicism and more accurate measurements of mosaicism frequency by real-time PCR could provide clearer view of the correlation between the frequency of mutant allele and disease severity.

The mechanism of LPS-induced monocyte death that we observed remains to be elucidated. When monocytes are treated with LPS, *CIAS1* mRNA is induced immediately, and its encoded protein cryopyrin can be detected within 30 to 60 minutes of treatment.[36] One simple possibility is that the accumulation of LPS-induced mutant cryopyrin in the cytosol mediates necrosis. This is supported by our recent observation that overexpression of a