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Competing interests statement

The authors declare that they have no competing financial interests.

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Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency

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Apoptosis is a form of programmed cell death that is controlled by aspartate-specific cysteine proteases called caspases. In the immune system, apoptosis counters the proliferation of lymphocytes to achieve a homeostatic balance, which allows potent responses to pathogens but avoids autoimmunity[1,2]. The CD95 (Fas, Apo-1) receptor triggers lymphocyte apoptosis by recruiting Fas-associated death domain (FADD), caspase-8 and caspase-10 proteins into a death-inducing signalling complex[3,4]. Heterozygous mutations in CD95, CD95 ligand or caspase-10 underlie most cases of autoimmune lymphoproliferative syndrome (ALPS), a human disorder that is characterized by defective lymphocyte apoptosis, lymphadenopathy, splenomegaly and autoimmunity[5–14]. Mutations in caspase-8 have not been described in ALPS, and homozygous caspase-8 deficiency causes embryonic lethality in mice. Here we describe a human kindred with an inherited genetic deficiency of caspase-8. Homozygous individuals manifest defective lymphocyte apoptosis and homeostasis but, unlike individuals affected with ALPS, also have defects in their activation of T lymphocytes, B lymphocytes and natural killer cells, which leads to immunodeficiency. Thus, caspase-8 deficiency in humans is compatible with normal development and shows that caspase-8 has a postnatal role in immune activation of naive lymphocytes.

Some individuals studied at the NIH under approved protocols manifest ALPS-related clinical features but do not have mutations in CD95, CD95 ligand or caspase-10 (ref. 15). Among them, we identified two siblings in family 66, a 12-yr-old female (patient 1) and an 11-yr-old male (patient 2), who showed lymphadenopathy, splenomegaly and defective CD95-induced apoptosis of peripheral blood lymphocytes (PBLs; Table 1 and Fig. 1a). Unlike typical individuals affected with ALPS, they had immunodeficiency characterized by recurrent sinopulmonary and herpes simplex virus (HSV) infections and poor responses to immunization (Table 1). The affected siblings were developmentally normal. The unaffected mother, father and sister were clinically well, although their PBLs showed partial defects in apoptosis mediated by CD95 (Fig. 1a and data not shown). Cells from patient 1 responded normally to the mitochondrial apoptosis inducers staurosporine, ceramide and etoposide (Fig. 1b).

Biochemical analysis of the death-inducing signalling complex (DISC) showed normal CD95-induced FADD recruitment in all members of family 66 examined (Fig. 1c and data not shown). By contrast, the cellular abundance of caspase-8 and its recruitment to the DISC were decreased markedly in patient 1 and patient 2, but were normal in the mother (Fig. 1c, d, and data not shown). Neither caspase-8 nor the downstream effector caspase-3 underwent cleavage after Fas ligation, indicating that the DISC from patient 1 cells was not functional (Fig. 1e). We sequenced the complementary DNAs of CD95, CD95 ligand, caspase-10 and FADD from family members but found no mutations; however, we identified a homozygous C to T mutation in caspase-8 in both patient 1 and patient 2 that changed an arginine to a tryptophan at residue 248 in the p18 protease subunit (Fig. 2a).

The asymptomatic mother, father and sister were heterozygous carriers of this mutation. We screened 13 extended family members and detected seven asymptomatic heterozygous carriers, but no additional homozygous or immunodeficient individuals. The pedigree revealed consanguinity (Fig. 2b). We did not find the mutation in DNA samples from 40 normal donors and 40 individuals affected with systemic lupus erythematosus, indicating that the Arg248Trp mutation is not a polymorphism. No additional caspase-8 mutations were found in 10 individuals with common variable immunodeficiency or 30 individuals with ALPS-like conditions (data not shown).

We examined the activity of the mutant enzyme. Using purified chimaeric proteins of wild-type or mutant caspase-8 protease fused to glutathione *S*-transferase (GST), we found that the mutant protein was unable to cleave the reporter substrate DEVD-AMC (Fig. 2c). In a cellular reconstitution assay using a caspase-8-deficient Jurkat cell line (I9.2) that fails to undergo apoptosis after CD95 crosslinking, expression constructs encoding wild-type caspase-8 restored CD95-induced apoptosis, but the mutant caspase-8

Table 1 **Phenotype of kindred that are genetically deficient for caspase-8**

Subject*	Patient 1	Patient 2	Mother 66	Sister 66
Total lymphocytes (832–2,028)	1,510	2,362	1,456	1,677
CD4 % (32.6–58.9)	23.1	25.0	42.3	36.2
CD8 % (17.8–46.7)	49.6	46.2	30.7	32.3
CD4/CD8 ratio (0.75–3.31)	0.5	0.5	1.4	1.1
B lymphocytes (88–330)	361	570	326	354
Clinical symptoms				
Failure to thrive/short stature	+	+	−	−
Lymphadenopathy	+	+	−	−
Splenomegaly	+	+	−	−
Eczema	+	+	−	−
Reactive airway disease	+	+	−	−
HSV labialis	+	+	−	−
Pneumonia	+	+	−	−
Asthma	+	+	−	−
Chronic diarrhoea	−	+	−	−
Response to pneumococcal immunization	−	−	ND	+
Immunoglobulin concentrations (mg dl^{-1})				
IgG (723–1,685)	857	544	ND	864
IgA (81–463)	112	56	ND	114
IgM (48–271)	52	32	ND	27
IgE (<180)	7	20	ND	28

*The normal range for each value is shown in parentheses. ND, not determined.

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did not (ref. 16 and Fig. 2d). In an apoptosis assay in which fusion to the CD8 ectodomain spontaneously drives the multimerization of caspase-8 (ref. 17), the wild-type construct (CD8–C8wt) induced cell death, whereas the mutant construct (CD8–C8mt) was inactive (Fig. 2e). Densitometry showed that the abundance of mutant caspase-8 protein in the homozygotes was less than 5% of the wild-type amount, indicating that the mutant protein is unlikely to be partially functional or have allosteric regulatory effects. Thus, the Arg248Trp mutation reduces the stability of the caspase-8 protein and renders it enzymatically inactive.

The association between the homozygous caspase-8 mutation and immunodeficiency led us to study lymphocyte activation responses. We found interleukin-2 (IL-2) production was markedly defective when PBLs from individuals homozygous for the mutant caspase-8 were stimulated through the T-cell receptor (TCR; Fig. 3a). Notably, the heterozygous family members had a partial defect in IL-2 release. IL-2 production was rescued by stimulation with phorbol myristate acetate (PMA) and ionomycin, which bypass proximal TCR signalling and induce second messengers directly (Fig. 3b). We also found diminished T-cell proliferation responses to phytohaemagglutinin (PHA) in the two homozygotes, whereas the heterozygotes showed minimal proliferative defects (Fig. 3c). CD4- and CD8-postive T-cell populations from patients 1 and 2 showed calcium defects, indicating that the defect in activation affected both subsets of T cells (Fig. 3d and data not shown).

We examined the influence of caspase-8 on surface activation markers after lymphocyte stimulation (Table 2). The basal surface expression of CD3, CD28 and CD95 was normal (data not shown); however, CD25 induction was markedly defective on both CD4- and CD8-positive T lymphocytes from patients 1 and 2. Induction defects were also observed for CD28, CD71, CD95, CD134, CD152 and MHC class II molecules, whereas induction of CD137 and downregulation of the TCR were not affected (Table 2 and data not shown). Cells from heterozygous family members also showed substantial defects in activation marker expression that were exacerbated by the caspase inhibitor zVAD-fmk (Table 2 and data

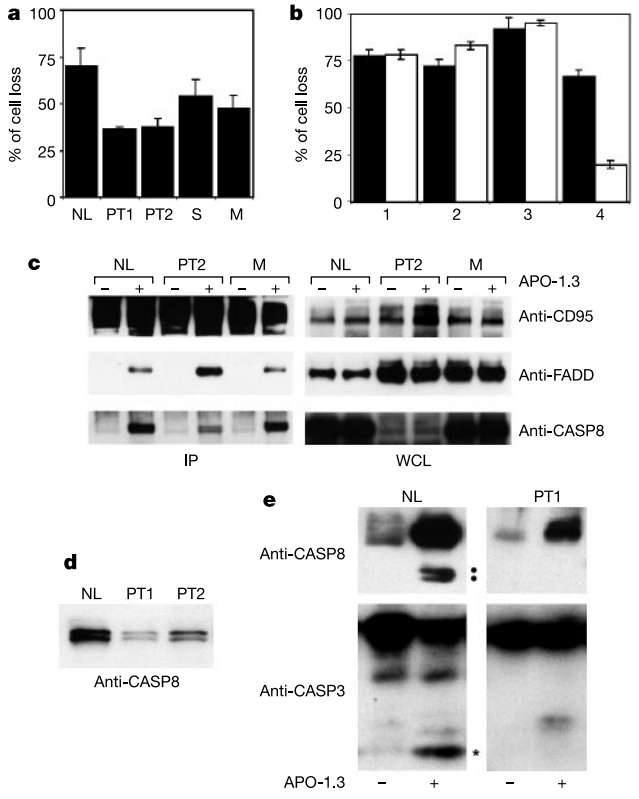

Figure 1 Defective apoptosis and DISC analysis in family 66. **a**, Activated PBLs from normal volunteers (NL), patient 1 (PT1), patient 2 (PT2), the sister (S) and mother (M) were stimulated with antibody to CD95 and analysed for cell loss. **b**, B cells transformed with the Epstein–Barr virus (EBV) were treated with staurosporine (1), etoposide (2), ceramide (3) or antibodies to CD95 (4) and analysed for cell loss. Filled bars represent normal control cells, open bars represent PT1 cells. **c**, DISC immunoprecipitates (IP) using antibodies to CD95 and whole-cell lysates (WCLs) from PBLs either treated (+) or untreated (−) with antibody to CD95 (APO-1.3) were blotted using antibody to CD95, FADD or caspase-8. **d**, Immunoblot showing amount of caspase-8 in PBL lysates. **e**, DISC immunoprecipitates from cells with or without anti-CD95 crosslinking were blotted with antibody to caspase-8 (top), and the corresponding lysates were blotted with antibody to caspase-3 (bottom). Cleavage products of caspase-8 and caspase-3 are marked by dots and an asterisk, respectively. Data are representative of four experiments.

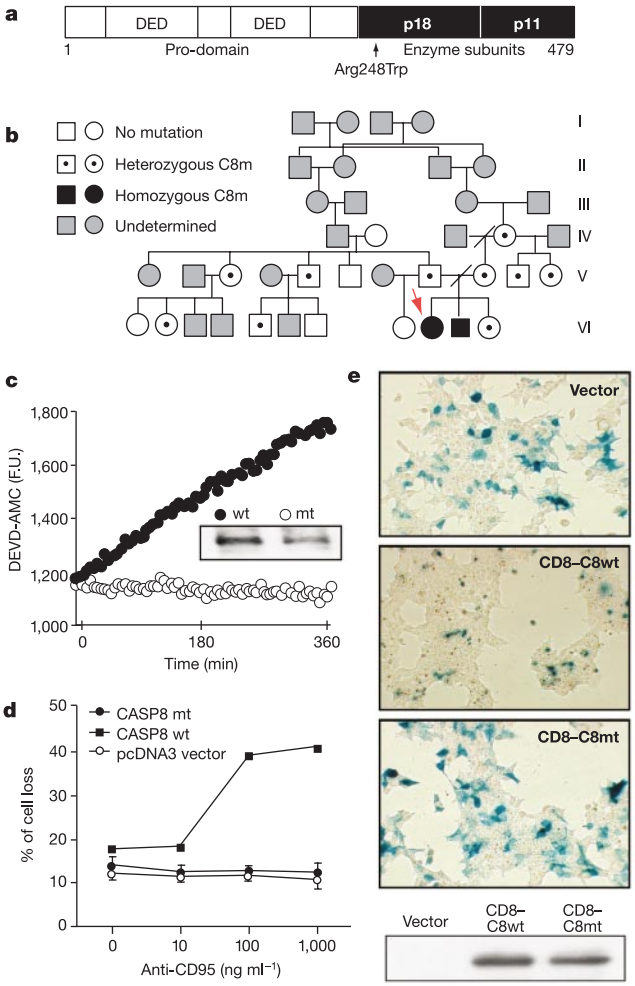

Figure 2 Characterization of an inherited caspase-8 mutation. **a**, Caspase-8 protein showing the death effector domains (DED) and the enzyme subunits (p18 and p11). The location and predicted amino acid substitution of the mutation are indicated by the arrow. **b**, Pedigree of family 66. Patient 1 is indicated by the red arrow. **c**, Measurement of DEVD-AMC cleavage using GST fusion proteins of wild-type (wt, filled circles) or Arg248Trp mutant (mt, open circles) caspase-8. Inset, immunoblot for caspase-8 shows that nearly equivalent amounts of the two GST–caspase-8 fusion proteins were used in the assay. F.U., arbitrary fluorescence units. **d**, Jurkat I9.2 cells were transfected with vector (open circles), wild-type caspase-8 (filled squares) or Arg248Trp mutant caspase-8 (filled circles), treated with antibody to CD95, and assessed for cell loss. **e**, 293T cell lines were transfected with vector (top), or with CD8 fusions of wild-type (middle) or mutant Arg248Trp (bottom) caspase-8 along with a β-galactosidase expression vector. The immunoblot of transfected cell lysates with antibody to caspase-8 shows similar expression of caspase-8 protein. Data are representative of three experiments.

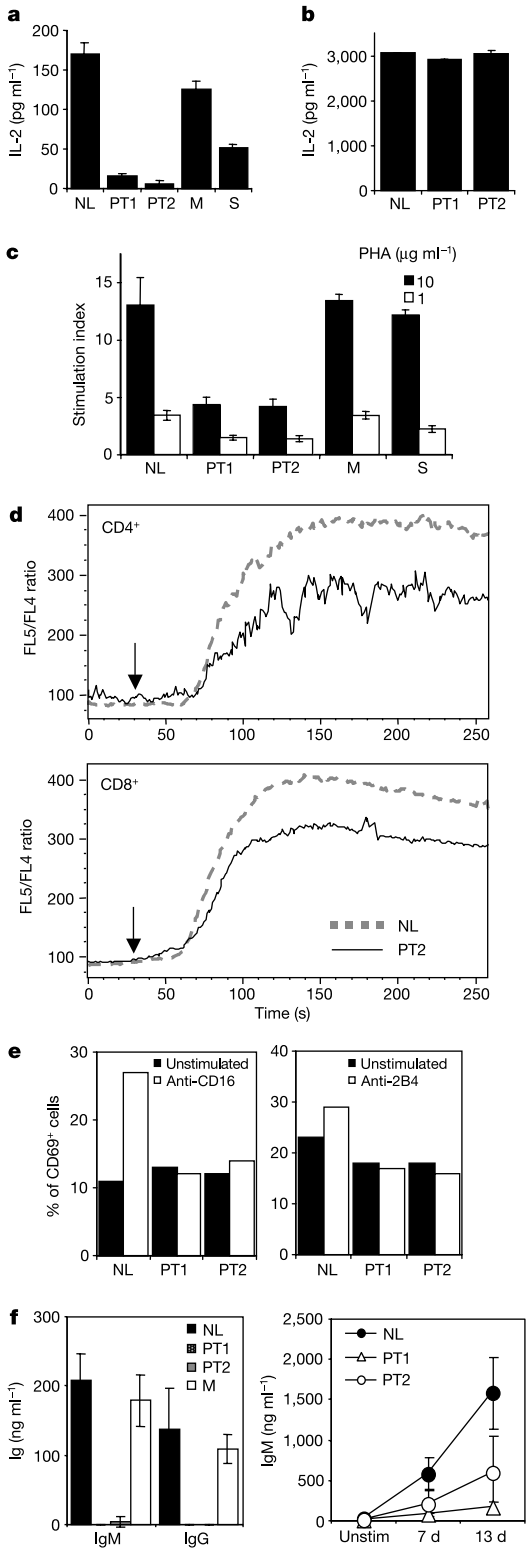

Figure 3 Defective immune cell responses in homozygous caspase-8 mutants. **a**, IL-2 release after stimulation with antibodies to CD3 and CD28 (10 μg ml^{-1} each) for family members abbreviated as in Fig. 1. **b**, IL-2 release after stimulation with PMA and ionomycin. **c**, Stimulation index measured by thymidine incorporation after treatment with PHA. **d**, Calcium flux in CD4$^+$ (top) or CD8$^+$ (bottom) cells after TCR and CD28 stimulation. **e**, CD69 expression in purified NK cells after stimulation with antibodies to CD16 (left) or to 2B4 (right). **f**, Production of immunoglobulins by PBLs that were unstimulated (unstim) or stimulated with pokeweed mitogen (PWM; left) or by purified B cells that were stimulated with SAC and IL-2 for the indicated number of days (right). Data are representative of four experiments.

not shown). By contrast, control experiments showed that lymphocytes from individuals with conventional ALPS and CD95 mutations did not have defective induction of surface activation molecules (data not shown). These results show that caspase-8 has a pivotal role in a proximal signalling event that is essential for a selected subset of TCR-induced responses.

A prominent feature of human homozygous caspase-8 deficiency is severe infection by mucocutaneous HSV. Because defects in natural killer (NK) cells predispose to herpes virus infections, we examined signalling by the CD16 and 2B4 pathways in purified NK cells[18–20]. Activation of NK cells manifested by CD69 expression was induced by stimulating the cells with antibodies to CD16 or 2B4 in the control samples, but not in samples from patients 1 or 2 (Fig. 3e). Defective NK cell activation by 2B4 could be reconstituted in normal controls by pretreatment with zVAD-fmk (data not shown). The variable decreases in serum immunoglobulin concentrations in these individuals also prompted us to test their B-cell function. We

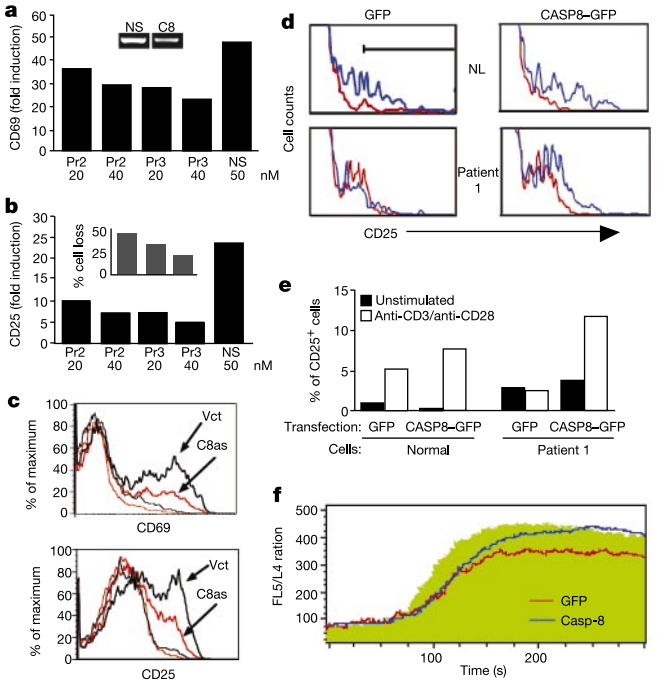

Figure 4 Caspase-8 is required for lymphocyte activation. **a**, Induction of the surface expression of CD69. Resting PBLs were transfected for 16 h with RNAi primers that were either nonspecific (NS) or specific for caspase-8 (primer pair 2 or 3), and were then stimulated with TCR and CD28 for 18 h. Inset shows that less caspase-8 mRNA is produced by RT–PCR with primer pair 3 (C8) than with the nonspecific primers (NS). **b**, Induction of the surface expression of CD25 analysed as in **a**. Inset shows a reduced percentage of cell loss after anti-CD95 antibody (500 ng ml^{-1}) stimulation of HeLa cells transfected with primer pair 2 (2) or primer pair 3 (3), as compared with nonspecific primers (1). **c**, Flow cytometry of PBLs co-transfected with 2 μg of pEGFP and 5 μg of vector (Vct, black) or a caspase-8 antisense construct (C8as, red) and cultured for 72 h before treatment with control medium (thin curves) or TCR and CD28 stimulation (1 μg ml^{-1} each, thick curves) for 6–24 h. Expression of CD69 at 6 h (top) or CD25 at 24 h (bottom) is shown. Protein blots show less expression of caspase-8 in samples transfected with C8as as compared with controls; the results in **a**–**c** were reproduced with eight random blood donors. **d**, Flow cytometry analysis of the surface expression of CD25 by normal (NL) and patient 1 PBLs transfected with GFP control (left) or wild-type caspase-8–GFP fusion protein (right) and either stimulated with antibodies to CD3 and CD28 (blue line) or left unstimulated (red line). Black line indicates the positive gate used for quantification. **e**, Representation of the results shown in **d**. **f**, Calcium flux after stimulation with antibodies to CD3 and CD28 in PBLs from normal controls (green area) or from patient 1, transduced with either GFP control (red line) or caspase-8–GFP retrovirus (blue line). Data are representative of three experiments.

found that PBLs stimulated with pokeweed mitogen (PWM), a T-cell-dependent mitogen, showed markedly decreased production of immunoglobulin-μ (IgM) and immunoglobulin-γ (IgG) in patients 1 and 2, whereas the mother showed normal production of immunoglobulins (Fig. 3f). IgM release was also impaired when purified B cells from patients 1 and 2 were stimulated with the T-cell-independent mitogen *Staphylococcus aureus* Cowan I (SAC) plus exogenous IL-2 (Fig. 3f). Thus, immunodeficiency and the resultant opportunistic infections associated with homozygous caspase-8 mutation reflect pleiotropic defects that involve the functions of T, NK and B cells.

We validated the association of caspase-8 deficiency with defective lymphocyte activation by two approaches. First, we inactivated caspase-8 in normal human peripheral blood lymphocytes with RNA interference (RNAi) and antisense techniques. Using an electroporation apparatus (Nucleofector System, Amaxa) for efficient gene transfection of resting human PBLs, we introduced two different duplex RNAi ribonucleotides specific for caspase-8 and then stimulated the cells through the TCR complex[21]. Induction of CD69 and CD25 showed that RNAi primer pairs 2 and 3 substantially reduced the response to TCR stimulation as compared with nonspecific primers (Fig. 4a, b). Controls showed that the specific primer pairs reduced caspase-8 messenger RNA and decreased CD95-induced apoptosis (Fig. 4a, b, insets). In addition, transfection of caspase-8 antisense constructs impaired CD69 and CD25 induction as compared with vector controls, further showing that interfering with expression of the caspase-8 gene blocks activation responses (Fig. 4c).

Second, we transfected lymphocytes from patient 1 with an expression construct that encoded either a wild-type fusion protein of caspase-8 and green fluorescent protein (GFP) or GFP alone. Cells transfected with the GFP control did not show upregulation of CD25 expression after stimulation, but cells transfected with the wild-type caspase-8–GFP construct showed a threefold or greater induction of CD25 expression, comparable to normal controls (Fig. 4d, e). Introduction of a functional caspase-8 molecule by lentiviral gene transduction also rescued the impaired calcium response to TCR stimulation in cells from patient 1 (ref. 22 and Fig. 4f). We also found that the caspase-8-deficient I9.2 Jurkat line manifested defects in activation responses that could be corrected by retroviral expression of the wild-type caspase-8 gene (Supplementary Information). Together, these findings show that caspase-8 is necessary for proximal signalling events in lymphocytes.

Our analysis of individuals with inherited caspase-8 deficiency has shown that caspase-8 has a broad role in the activation of T, B and NK cells in addition to its function in conveying signals from death receptors to apoptosis effector mechanisms[12,13]. Our results also explain how chemical caspase inhibitors interfere with T-cell proliferation[12–14]. Caspase-8 probably functions in lymphocyte activation independently of CD95, because individuals with type I ALPS and defects in CD95 do not have immunodeficiency, which makes it unlikely that this ligand–receptor pair is involved[23]. The immunodeficiency associated with defective caspase-8 also contrasts with the autoimmune phenotype of individuals with caspase-10 deficiency, despite the structural homology of these two enzymes[11]. Because mice that are deficient in caspase-8 show embryonic lethality, humans that lack caspase-8 function might not be expected to survive. Postnatal survival in humans may be due to the function of caspase-10, the closest paralogue of caspase-8 in humans. Caspase-10 has no known orthologue in mice but may compensate for caspase-8 (refs 3, 4). The phenotypes associated with caspase-8 and caspase-10 mutations in humans are based on limited genetic data, and studying more individuals with these disorders may define better the different consequences of these mutations. The defects in activation of T, NK and B cells suggest that there may be a common locus of action for caspase-8 in several lymphocyte signalling pathways that will be interesting to uncover. Caspase-8 may be a potentially useful target for a new class of anti-inflammatory or immunosuppressive therapeutics and there may be non-apoptotic signalling roles for caspases in cellular responses of non-immune organ systems. Hence, caspase-8 establishes a close link between the biochemical pathways of cellular activation and apoptosis that may shed light on the homeostatic regulation of these seemingly conflicting processes. □

Table 2 **Fluorescence of surface markers after CD3/CD28 antibody stimulation**

Surface markers	Normal (+/+)	Patient 1 (−/−)	Patient 2 (−/−)	Normal + 50 μM zVAD-fmk
CD25/CD4	514	42	97	18
CD25/CD8	417	23	112	10
CD28	510	178	316	170
CD71	204	30	64	16
CD95	141	39	60	54
CD134	62	22	50	13
CD137	20	17	44	6
CD152	26	12	16	13
MHC class II molecules	120	45	47	23

Geometric mean fluorescence of surface activation molecules after stimulation with antibodies to CD3 and CD28. The geometric mean fluorescence of each marker was calculated from the CD3-positive populations (or CD4- and CD8-positive populations for CD25). Unstimulated cells had similar values of geometric mean fluorescence. Data are representative of ten experiments.

Methods

Cellular preparations and analysis

Individuals were studied under NIH-approved ALPS research protocols with informed consent. Wild-type (JA3) and caspase-8-deficient (I9.2) Jurkat cells were gifts from J. Blenis. The 293T cell line was obtained from ATCC. Peripheral blood T lymphocytes were separated on Ficoll gradients and stimulated first with 5 μg ml^{-1} of PHA (Sigma) for 3 d and then with 100 IU ml^{-1} IL-2. Apoptosis assays used 10–1,000 ng ml^{-1} antibodies to CD95 (APO-1.3, Kamiya), 0.1 μg ml^{-1} staurosporine, 10 μg ml^{-1} etoposide or 10 μM ceramide as described[5,24]. We purified B cells using fluorescein isothiocyanate (FITC)-labelled antibodies to CD19, followed by positive selection with anti-FITC Microbeads (MACS). We prepared NK cells by negative selection using the NK Cell Isolation Kit (MACS). Immunoprecipitation and western blot analyses were carried out using antibodies to caspase-3 and FADD (Transduction Laboratories), CD95 (Kamiya) and caspase-8 (a gift from M. Peter).

Sequence analysis, plasmids and mutagenesis

We purified RNA using Trizol (Gibco-BRL) and carried out polymerase chain reaction with reverse transcription (RT–PCR) using the OneStep RT–PCR kit (Qiagen). Sequence analysis was done by dye primer chemistry (Amersham) on an ABI 377 automated sequencer and also by Cleveland Genomics. Bacterially expressed GST fusion proteins, full-length caspase-8 fused to GFP at the carboxy terminus for transfection studies, and CD8–caspase-8 constructs were prepared as described[11,17]. We generated mutations using the QuickChange Site-Directed Mutagenesis kit (Stratagene).

Biochemical and functional assays

Purification of GST fusion proteins and caspase assays using DEVD-AMC have been described[11]. We transfected I9.2 cells with various constructs and a GFP control vector at a molar ratio of 3:1. After 18 h, cells were treated with antibody to CD95 and assayed for apoptosis. Semiconfluent 293T cells or HeLa cells (RNAi) were transfected using Fugene 6 (Roche) according to the manufacturer's instructions with 2 μg of caspase-8 constructs and 1 μg of a 3LacZ construct. Cells were then fixed and stained for β-galactosidase as described[17]. To transfect resting PBLs, we mixed 5 μg of DNA containing the GFP fusion proteins with 100 μl of the human T-cell Nucleofector solution and then used this to resuspend 5×10^6 PBLs that had been freshly isolated using Ficoll. The PBL suspension was immediately electroporated by a Nucleofector instrument (Amaxa Biosystems). The transfected cells were added to 1 ml of complete RPMI-1640 medium for 24 h before being exposed to plate-bound anti-CD3 and anti-CD28 antibodies (each at 10 μg ml^{-1}, Pharmingen) for 24 h.

For RNAi analysis, primer pair 2 (5′-AATCACAGACTTTGGACAAAG-3′ and 5′-AACTTTGTCCAAAGTCTGTGA-3′, starting at nucleotide 653 from caspase-8 ATG), primer pair 3 (5′-AACTACCAGAAAGGTATACCT-3′ and 5′-AAAGGTATACCTTTC TGGTAG-3′, starting at nucleotide 1,090 from caspase-8 ATG) and nonspecific (NS) primers (5′-AACACGTAGCAGCTCGGATCG-3′ and 5′-AACGATCCGAGCTGCTAC GTG-3′) were used in the Silencer siRNA construction kit (Ambion). The caspase-8 primers used in RT–PCR were FLICE Fr (5′- CCTTGGGAATATTGAGATTATATTCTCC-3′) and FLICE Rev (5′-ATAGCACCATCAATCAGAAGGGAAGACAAG-3′). We generated the antisense construct by inserting the 498-base-pair *Eco*RI/*Xma*I caspase-8 cDNA fragment including the translation start site into a pEGFP-C1 vector (Clontech). The expression of CD25 and CD69 was determined by staining the surface of cells with the corresponding phycoerythrin-conjugated antibodies (Pharmingen). We analysed the data after gating on live GFP-positive cells.

Stimulation, cytokine measurements and proliferation assays

Resting PBLs were stimulated for typically 24 h with 1 μg ml^{-1} anti-CD3 and 5 μg ml^{-1} anti-CD28 antibodies (Pharmingen) or with 5 μg ml^{-1} PHA. We stimulated NK cells with 50 μg ml^{-1} of either plate-bound anti-CD16 (3G8) or anti-2B4 (C1.7) antibodies (Pharmingen) for 24 h. B lymphocytes were stimulated with either 10 μg ml^{-1} pokeweed mitogen (Sigma) or 10% (v/v) of SAC cells (Calbiochem) and 200 IU ml^{-1} of IL-2. Surface molecules of interest were detected using phycoerythrin (PE)- and FITC-labelled antibodies (Pharmingen) by standard procedures, and analysed on a FACScan flow cytometer (Becton-Dickinson) using CellQuest (Applied Biosystems) and FlowJo (TreeStar) software. Concentrations of immunoglobulin in the supernatants of cells at days 7 and 13 were measured by R. Hornung at the Immunological Monitoring Laboratory of the National Cancer Institute. For stimulations with PMA and ionomycin, 10 ng ml^{-1} and 0.5 μg ml^{-1} were used, respectively. Where indicated, cells were pretreated for 36 h with 50 μM zVAD-fmk (Enzyme Systems Products). We measured IL-2 after 48 h of stimulation using the Quantikine Immunoassay Kit (R&D Systems). In proliferation assays, cells stimulated for 48 h with PHA and anti-CD28 antibody were pulsed with tritiated thymidine for 24 h and assessed by scintillation counting.

Luciferase and calcium flux assays

Jurkat T cells were transfected with 10 μg of an IL-2–Luc construct and 0.2 μg of a renilla construct as described[25], stimulated with 20 μg ml^{-1} of plate-bound anti-CD3 and anti-CD28 antibodies for 24 h, and analysed with the Luciferase Assay System (Promega). To measure intracellular calcium, we loaded PBLs with 3 mM Indo-1 acetoxymethyl ester (Indo-1, Molecular Probes), incubated them at 37 °C and then stimulated them with 1 μg ml^{-1} of anti-CD3 and 5 μg ml^{-1} of anti-CD28 antibodies for about 6 min. The Indo-1 ratio of 395 nm/500 nm fluorescence emission was calculated by flow cytometry.

Retroviral infections

Lentiviral transduction of PBLs used plasmids that contained, in order, the HIV long terminal repeat (LTR), the caspase-8 coding sequence, an internal ribosome entry site (IRES) and GFP (HIV-LTR–caspase-8–IRES–GFP) or HIV-LTR–IRES–GFP, together with a packaging vector (Δ8.2) and a CMV–VSV-G construct (a gift from F. Candotti)[22] or the kat system[26]. We transfected the lentiviral vectors into 293T cells to generate viral supernatants, which we then concentrated by spinning.

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A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*

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Localized attack by a necrotizing pathogen induces systemic acquired resistance (SAR) to subsequent attack by a broad range of normally virulent pathogens. Salicylic acid accumulation is required for activation of local defenses, such as pathogenesis-related protein accumulation, at the initial site of attack, and for subsequent expression of SAR upon secondary, distant challenge[1,2]. Although salicylic acid moves through the plant, it is apparently not an essential mobile signal[2]. We screened *Agrobacterium tumefaciens* transfer DNA (tDNA) tagged lines of *Arabidopsis thaliana* for mutants specifically compromized in SAR. Here we show that *Defective* in *induced resistance 1-1* (*dir1-1*) exhibits wild-type local resistance to avirulent and virulent *Pseudomonas syringae*, but that pathogenesis-related gene expression is abolished in uninoculated distant leaves and *dir1-1* fails to develop SAR to virulent *Pseudomonas* or *Peronospora parasitica*. Petiole exudate experiments indicate that *dir1-1* is defective in the production or transmission from the inoculated leaf of an essential mobile signal. *DIR1* encodes a putative apoplastic lipid transfer protein and we propose that

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