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# Inherited Human Caspase 10 Mutations Underlie Defective Lymphocyte and Dendritic Cell Apoptosis in Autoimmune Lymphoproliferative Syndrome Type II

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## Summary

**Caspases are cysteine proteases that mediate programmed cell death in phylogenetically diverse multicellular organisms. We report here two kindreds with autoimmune lymphoproliferative syndrome (ALPS) type II, characterized by abnormal lymphocyte and dendritic cell homeostasis and immune regulatory defects, that harbor independent missense mutations in *Caspase 10*. These encode amino acid substitutions that decrease caspase activity and interfere with death receptor–induced apoptosis, particularly that stimulated by Fas ligand and TRAIL. These results provide evidence that inherited nonlethal caspase abnormalities cause pleiotropic apoptosis defects underlying autoimmunity in ALPS type II.**

## Introduction

A dynamic balance in the quantity and activation state of cells participating in immune responses is essential for achieving appropriate proliferative and effector responses to antigens while avoiding autoimmunity (for reviews, Abbas, 1996; Lenardo, 1997). This has been best defined for lymphocytes for which removal of T or B cells by antigen receptor engagement or the loss of trophic stimuli leads to apoptosis during and after peripheral immune responses, thereby allowing feedback or "propriocidal" regulation (Thompson, 1995; Lenardo, 1996). Antigen-provoked death of lymphocytes is mediated by Fas (CD95/APO-1), tumor necrosis factor receptor (TNFR), and related molecules (Russell and Wang, 1993; Zheng et al., 1995; Chinnaiyan and Dixit, 1997). Other death ligands such as TRAIL and its corresponding death receptors DR4 and DR5 are expressed in immune cells, but their physiological functions are not well understood (Wiley et al., 1995; Marsters et al., 1996; Pitti et al., 1996; MacFarlane et al., 1997; Pan et al., 1997; Sheridan et al., 1997; Thomas and Hersey, 1998). Recent experiments suggest that the fate of specialized antigen-presenting cells is also regulated during immune responses. Ingulli et al. (1997) have shown that antigen-bearing dendritic cells directly interact with antigen-specific T cells, resulting in T cell activation and disappearance of the dendritic cells. Because dendritic cells are potent stimulators of B cells and T cells, their turnover may be essential for regulating immune responses (Banchereau and Steinman, 1998). Currently little is known about how this process is regulated.

We and others have identified the autoimmune lymphoproliferative syndrome (ALPS) as an inherited disease of lymphocyte homeostasis and defective apoptosis (Fisher et al., 1995; Rieux-Laucat et al., 1995; Drappa et al., 1996; Bettinardi et al., 1997; Sneller et al., 1997; Kasahara et al., 1998). ALPS patients manifest lymphocytosis, including an otherwise rare population of $CD4^-CD8^-$ T cells, and autoimmune disorders. In ALPS type Ia, this phenotype is associated with inherited mutations in the *APT1* gene on chromosome 10q24.1 encoding Fas (Straus et al., 1999). In ALPS type Ib, systemic lupus erythematosus and lymphadenopathy are associated with inherited mutations in the Fas ligand (FasL) gene on chromosome 1q23 (Wu et al., 1996). The *lpr* and *gld* alleles cause similar diseases in mice due to homozygous recessive mutations in the Fas and FasL genes, respectively (Watanabe-Fukunaga et al., 1992; Lynch et al., 1994; Takahashi et al., 1994). ALPS type II is manifested by a related clinical phenotype and apoptosis defects in the absence of either Fas or FasL mutations (Dianzani et al., 1997; Sneller et al., 1997). The molecular basis for ALPS type II has not been previously identified.

A key cell death molecule, CED-3, in *C. elegans* is the prototype of a new family of cysteinyl-aspartate-requiring proteinases, or caspases, that play a crucial role in mammalian apoptosis (Yuan et al., 1993; Horvitz et al., 1994; Alnemri et al., 1996; Nicholson and Thornberry, 1997). Caspases are synthesized as zymogens that are proteolytically activated either by autoprocessing or via other caspases. Caspase 8 (CASP8) occupies a proximal position in Fas and TNFR signaling because it contains two death effector domains (DEDs) in its N-terminal prodomain. The DEDs allow CASP8 to interact with the DED of Fas-associated protein with a death domain (FADD)/MORT1. Homotypic interaction of the death domains in FADD and death receptors thus allows CASP8 to be recruited into the death-inducing signaling complex (Boldin et al., 1996; Muzio et al., 1996; Juo et al., 1998). Deficiency in CASP8 causes prenatal mortality and a loss of TNF- and Fas-induced apoptosis in mice (Varfolomeev et al., 1998). Caspase 10/Mch4/FLICE2 (CASP10) is a novel caspase homologous to CASP8 and its gene linked to the *CASP8* gene at the human chromosome locus 2q23 (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997). CASP10 induces apoptosis after transfection into MCF-7 cells (Vincenz and

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Dixit, 1997), but its physiological role is unknown. Moreover, the participation of individual caspases in abnormalities of lymphocyte homeostasis and autoimmunity has not been defined.

Since the initial description of defective lymphocyte apoptosis associated with Fas mutations, we have examined 68 kindreds affected with various autoimmune and lymphoproliferative abnormalities, most of which prove to be ALPS type 1a. Here, we describe two ALPS type II families with autoimmunity and pleiotropic apoptosis defects in multiple pathways, but no molecular abnormalities in Fas, FasL, TNFR1, TNFR2, FADD, or CASP8. Rather, we found distinct inherited amino acid substitutions in CASP10 in each family that resulted in decreased enzyme activity and diminished apoptosis by multiple death receptors. In contrast to Fas mutations leading to ALPS type I, these *CASP10* defects underlie a unique disorder of dendritic cell and lymphocyte homeostasis.

## Results

### Clinical Features

The proband in family 11 is an 11-year-old African American female first evaluated at the NIH in 1992. At one year of age, she developed prominent nonmalignant adenopathy, hepatosplenomegaly, and Coombs-positive hemolytic anemia. She exhibited a wide-ranging loss of self-tolerance involving hypergammaglobulinemia with multiple autoantibodies, such as anti-erythrocyte, anti-RNP, anti-SM, anti-SSB, and rheumatoid factor as well as anti-Factor VIII antibody, which caused a severe clotting disorder. She had massive accumulation of single-positive T cells and B cells, indicating loss of lymphocyte homeostasis. Also, 42% of her peripheral lymphocytes were the normally rare ($<$1%) CD4$^-$CD8$^-\alpha\beta$ T cells, which are increased in ALPS type I and in murine *lpr* disease but usually not to this degree. The patient's mother exhibited high levels of autoantibodies to nuclear antigens and defective lymphocyte apoptosis (see below), both of which are features of ALPS. The patient's father and two sisters are healthy.

The proband of family 36 is a 10-year-old Ashkenazi Jewish male recently evaluated at the NIH. Adenopathy began at 11 months followed by bouts of prolonged fever, splenomegaly, elevated sedimentation rate, anemia, and reticulocytosis. At age 3, he had noninfectious, lymphocytic meningitis followed by optic neuritis indicating a pattern of disparate inflammatory conditions. At his recent evaluation, he exhibited adenopathy and splenomegaly. Autoantibodies were not detected, but lymphocyte phenotyping showed a dramatic T and B lymphocytosis and increased CD4$^-$/CD8$^-$ T cells, especially a striking increase in $\gamma\delta$ CD4$^-$/CD8$^-$ T cells. Both parents were clinically normal. Thus, despite normal Fas and FasL, both families manifested a loss of lymphoid homeostasis accompanied by autoimmune and inflammatory conditions.

### Lymphocyte Apoptosis Defects in ALPS Type II

In ALPS type I, T cell apoptosis induced by Fas or TCR cross-linking is abnormal (Fisher et al., 1995). We therefore examined apoptosis in activated peripheral blood T lymphocytes (PBL) in kindreds 11 and 36. In patient (Pt) 11 and, to a lesser extent, her mother, apoptosis induced by TCR cross-linking and Fas cross-linking was decreased (Figure 1). We also examined apoptosis responses to a trimeric form of the TRAIL death-inducing ligand (Walczak et al., 1999) and found defects in Pt 11 and her mother's lymphocytes, but not in ALPS type I patients (Figure 1 and data not shown). No apoptosis defects were found in the father and sisters of Pt 11 (Figure 1). Pt 36 also exhibited abnormal apoptosis responses to TCR, Fas, or TRAIL stimulation, while his parents exhibited modest but significant defects. Flow cytometry showed that Fas expression as well as apoptosis in response to staurosporine or lymphokine withdrawal was normal in Pt 11 and Pt 36 (data not shown). These data suggested that Pt 11 might have inherited a novel selective apoptosis defect from her mother in a heterozygous dominant fashion. By contrast, a putative genetic defect in Pt 36 would either have homozygous inheritance or have arisen de novo. Moreover, the apoptosis defects in these families, unlike that in ALPS type I, affect death receptors in addition to Fas.

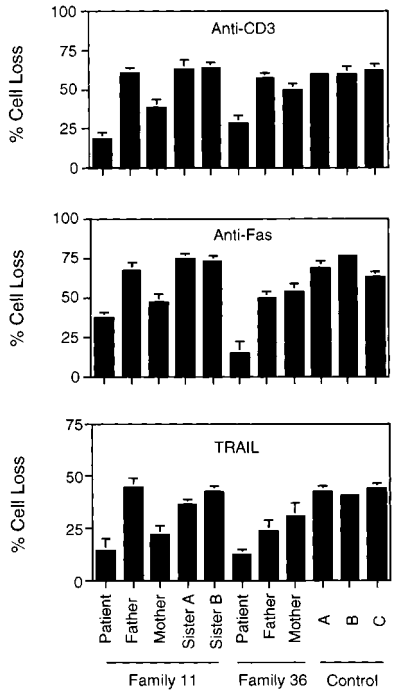


Figure 1. Lymphocyte Apoptosis Assays

Apoptosis of PBLs were induced by 1 $\mu$g/ml plate-bound 64.1 (Anti-CD3), 1 $\mu$g/ml CH11 (Anti-Fas), or 1 $\mu$g/ml TRAIL at 37°C for 24 hr, and percent cell loss was analyzed as described (Fisher et al., 1995).

### Identification of CASP10 Mutations

To identify the molecular defect in Pt 11 and her mother, we examined candidate genes. Single-stranded conformational polymorphism (SSCP) analysis and sequencing of cDNAs revealed no mutations in the coding sequences of Fas, FasL, TNFR I (p55), FADD/MORT1, TRADD, RIP, CASP7, CASP8, or CASP9 (data not shown). However, we found a *CASP10* mutation in Pt 11 that was a C to T transition predicted to replace a

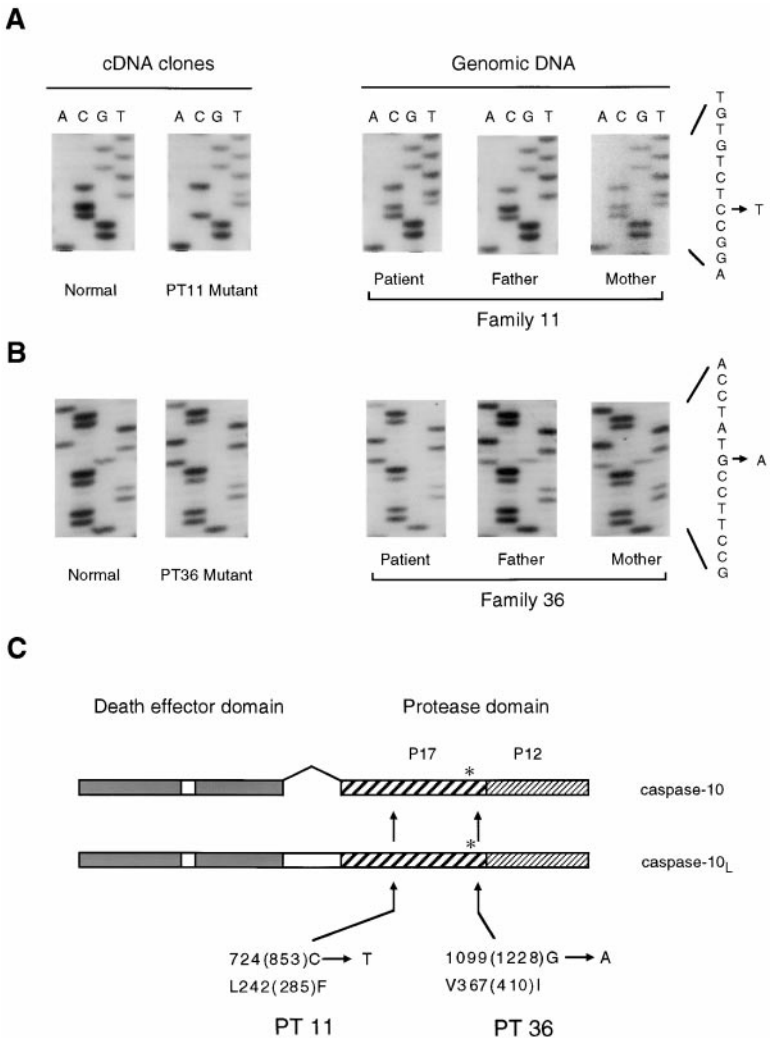


Figure 2. Sequences of CASP10

(A) Wild-type and Pt 11 mutant cDNA clones (left); family 11 genomic DNA (right).

(B) Wild-type and Pt 36 mutant cDNA clones (left); family 36 genomic DNA (right).

(C)The asterisk indicates the "QACQG" active site.

leucine by phenylalanine in the p17 subunit of the protease (Figure 2 and data not shown). In addition to the previously described *CASP10* cDNA (Fernandes-Alnemri et al., 1996), we also detected a longer cDNA isoform (*CASP10*$_L$). The *CASP10*$_L$ isoform encoded an insertion of 43 amino acids at the end of the prodomain identical to the prodomain of FLICE2, but its C terminus was the same as the short *CASP10* isoform (Vincenz and Dixit, 1997). The two isoforms were expressed equally. Approximately 50% of *CASP10* cDNAs (of either isoform) were mutant, implying heterozygosity. This was confirmed by direct sequencing of genomic DNA, which revealed the wild-type C and mutant T nucleotides at the same position (Figure 2A). Genomic DNA sequencing in the parents revealed that the father of Pt 11 and two sisters carried only wild-type alleles, but her mother harbored both mutant and wild-type alleles (Figure 2A and data not shown). Thus, the mutant *CASP10* allele in Pt 11 was inherited from her mother. No other base change in the coding or splicing sequences of *CASP10* was found.

In Pt 36, SSCP screening followed by sequencing of cDNA clones revealed a G to A transition predicted to cause a valine to isoleucine change seven amino acids downstream of the QACQG active site in the p17 subunit of the *CASP10* gene (Figure 2B). This mutation occurred in cDNAs of both isoforms of *CASP10*. In contrast to Pt 11, no wild-type allele was detected in Pt 36, suggesting that the mutant allele is homozygous (Figure 2B). This was confirmed by genomic DNA sequencing (Figure 2B). Examination of the genomic DNA of both parents revealed that each was heterozygous for the mutant allele, indicating that the child had indeed inherited a mutant *CASP10* allele from each parent. No other nucleotide changes were found in the *CASP10* gene in Pt 36 or his parents.

To rule out the possibility that the mutations in Pt 11 and Pt 36 represented common genetic polymorphisms, we analyzed their frequency in the general population and determined their effects on caspase function. The Pt 11 mutation destroyed a Stu I restriction site which we used to determine that 100 unrelated normal donor DNA samples, comprising 200 chromosomes, did not harbor this nucleotide change (data not shown). SSCP analysis did not detect the Pt 36 mutation in 120 genomic DNA samples (240 alleles) from healthy unrelated controls of varied ethnicity and 100 unrelated Ashkenazi Jewish genomic DNA samples (200 alleles) (data not

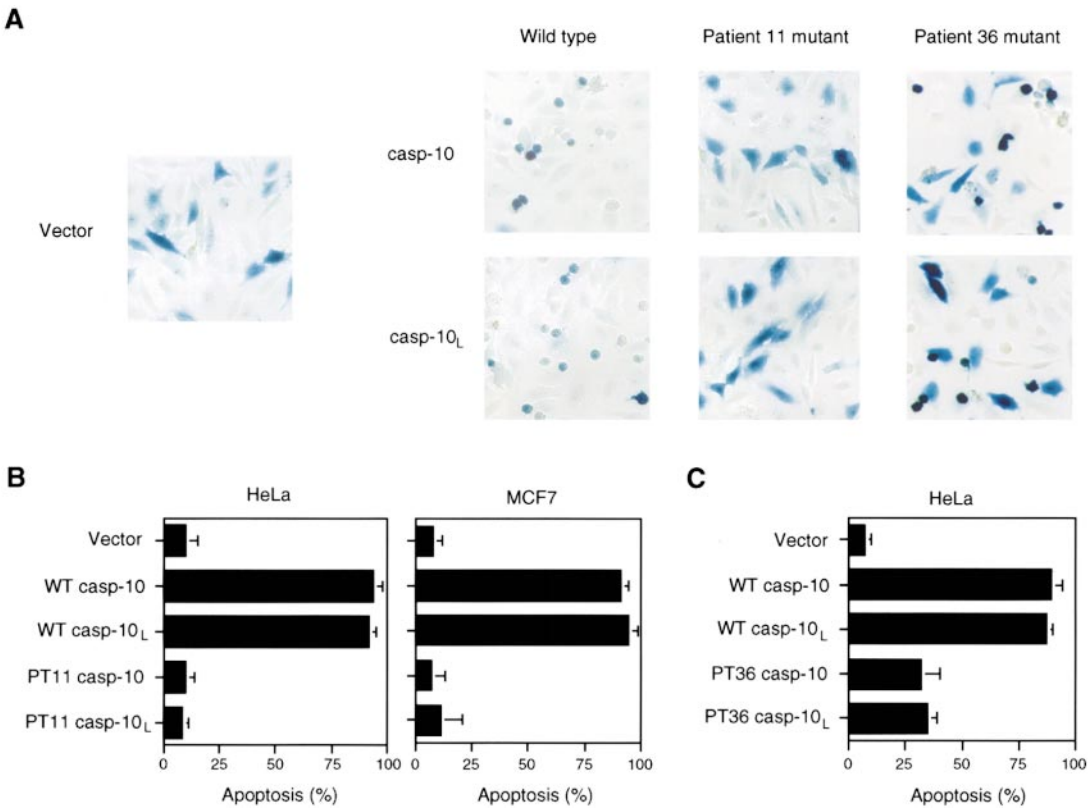


Figure 3. Defective Apoptotic Activities of Mutant CASP10

(A) HeLa cells were transfected with 2 μg CASP10 plasmid or vector only in the presence of 0.6 μg β-gal construct DNA. Cells were fixed and stained with X-gal 24 hr after transfection.

(B and C) Quantitation of apoptotic HeLa or MCF-7 cells after transfection with 2 μg wild-type or mutant CASP10 plasmids or vector only in the presence of 0.6 μg β-gal construct.

shown). The *CASP10* mutations have not been found in any ALPS type I patient or family member. Our screening did uncover one common polymorphic variant of *CASP10* (nucleotide A1208→G in the p12 subunit), which was observed in 5% of normal controls and had no effect on apoptotic function of CASP10 (data not shown). Therefore, the *CASP10* mutations in families 11 and 36 are not common genetic polymorphisms.

### Abrogation of Apoptosis Activities by CASP10 Mutations

The cooccurrence of *CASP10* mutations with apoptosis defects and immunological abnormalities in Pt 11 and Pt 36 suggested, but did not prove, that the mutations contributed to these abnormalities. We therefore determined whether the altered forms of CASP10 were functionally defective. Expression constructs of both the long and short isoforms of the wild-type or the CASP10 mutants were transfected into HeLa or MCF-7 cells together with a β-gal reporter construct. The cells were stained with X-gal, and cell death was assessed in the blue transfected cells (Figure 3). Either isoform of wild-type CASP10 induced significant cell death, but neither isoform of the Pt 11 had detectable apoptotic activity in this assay (Figures 3A and 3B). Similar analyses of the Pt 36 CASP10 revealed a significant, albeit less severe, defect in apoptosis function (Figures 3A and 3C).

Quantitative analysis was performed with the bacterially expressed CASP10 protease domain of Pt 11 fused to glutathione S-transferase (GST). The wild-type CASP10 efficiently cleaved DEVD-AMC and to a lesser extent YVAD-MCA, but the Pt 11 mutant protein failed to do so (Figure 4A). We also examined autoprocessing and found that the wild-type 55 kDa GST fusion protein was cleaved to the 43 kDa active form by 15 min, whereas the mutant protein showed only 50% cleavage at 3 hr (Figure 4B). To distinguish between decreased intrinsic catalytic ability versus the failure of the mutant protein to be recognized as an autosubstrate, equivalent amounts of cleaved wild-type and mutant GST-CASP10 were assayed using a DEVD peptide substrate (Figure 4C). We observed significantly reduced catalytic activity by the mutant enzyme, with a $K_m$ of 709 ± 14.4 μM compared to a $K_m$ of 71 ± 12.9 μM for the wild-type CASP10.

### Dominant-Negative Interaction of the Mutated CASP10

To explore how the heterozygous allele in Pt 11 and her mother could impair apoptosis, we coexpressed Fas with either the long or short isoform of the Pt 11 mutant CASP10 in 293 cells and found that apoptosis was suppressed (Figure 5). We also coexpressed the CASP10 mutants with the TNFR1, DR3, DR4 (TRAIL-R1), and DR5

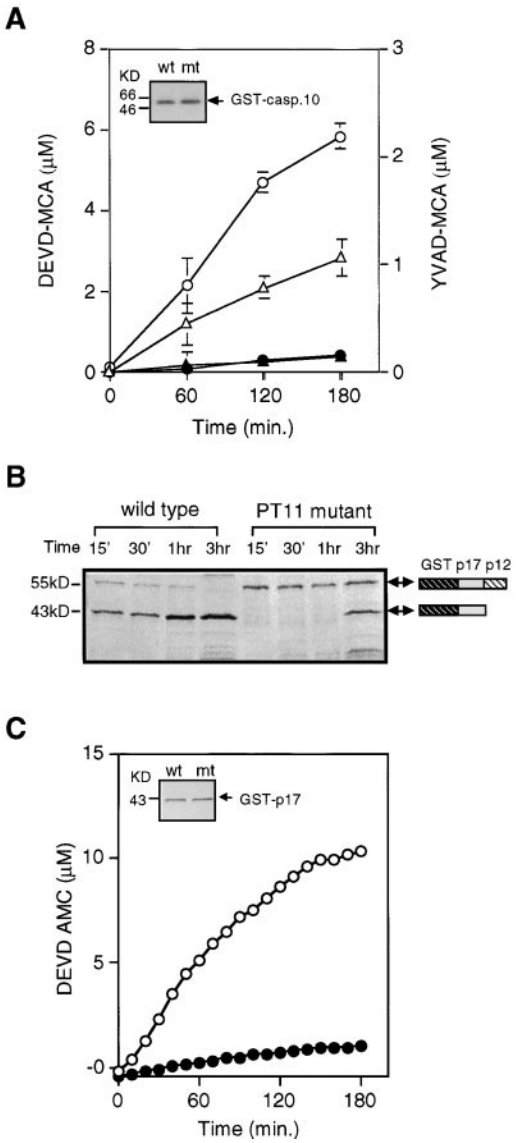


Figure 4. Decreased Ezymatic Activity of Pt11 CASP10

(A) Bacterially expressed GST-CASP10 attached to glutathione-conjugated beads was used for a cleavage assay with 50 μM DEVD-AMC (circles) or YVAD-AMC (triangles) substrate for 0, 1, 2, and 3 hr, and the release of AMC was quantitated by a fluorospectrophotometer (open symbols, wild type; filled symbols, mutant).

(B) GST-CASP10 attached to glutathione-conjugated beads was eluted with glutathione and incubated in protease buffer at 37°C for various times and then analyzed by SDS–PAGE.

(C) GST-CASP10 was prepared as in (A) and then allowed to fully process (using longer incubation times for the mutant compared to the wild-type CASP10) to the cleaved form and then used for enzyme assays with 50 μM DEVD-AMC substrate for various times. The release of AMC was quantitated by a fluorospectrophotometer (open symbols, wild type; filled symbols, mutant).

(TRAIL-R2) receptors, and in each case, the mutant caspase interfered with death induction (Figure 5). The p35 inhibitor blocked apoptosis in all cases, indicating that the death observed was caspase dependent. By contrast, the mutant CASP10 did not interfere with apoptosis of transfected cells treated with UV irradiation or staurosporine (data not shown). Hence, the abnormal CASP10 protein specifically interfered with multiple death receptor pathways and not other apoptosis pathways.

To explain the interference in death pathways caused by the heterozygous CASP10 mutation in Pt 11, we hypothesized that CASP10 might be recruited into death-inducing signaling complexes (DISCs) with the cytoplasmic tails of death receptors. Because the prodomain of CASP10 is structurally similar to that of CASP8, it is possible that the mutant CASP10 can be recruited along with wild-type CASP8 or CASP10 into the DISC. We first tested the interactions of CASP10 with CASP8 or FADD using the yeast two-hybrid system and found that the long and short isoforms of either full-length CASP10 or the CASP10 DEDs interact with both CASP8 and FADD (data not shown). To directly test recruitment of Pt 11 mutant CASP10 into the DISC, we cotransfected Fas, FADD, and both isoforms of mutant CASP10 into 293T cells. Both FADD and the mutant CASP10 were readily detected in coprecipitates with Fas, consistent with previous observations on FLICE2 (Figure 6; Vincenz and Dixit, 1997). Importantly, the mutant CASP10 isoforms are corecruited with endogenous CASP8 (Figure 6, bottom). This suggests the possibility that the mutant CASP10 from Pt 11, which inefficiently autoprocesses, is recruited into the DISC together with CASP8 and could thereby block caspase activation in a dominant fashion.

### Resistance of Dendritic Cells to TRAIL-Mediated Apoptosis

To explain the multiple autoimmune and inflammatory disorders in ALPS type II, we also examined how the Pt 11 and Pt 36 *CASP10* mutations affect death receptor–associated apoptosis in antigen-presenting cells. Previous observations of T cell–dependent turnover of dendritic cells suggested that dendritic cells may undergo apoptosis triggered by death receptors (Ingulli et al., 1997). Dendritic cells were derived from peripheral blood using a previously described procedure (Sallusto and Lanzavecchia, 1994), and we first asked whether activated T cells could foster their death in vitro. Using dendritic cells as chromium-labeled targets, we found that activated autologous $CD4^+$ T cells killed $CD83^+$ dendritic cells following TCR engagement (Figures 7A and 7B). The T cell–dependent lysis of dendritic cells was suppressed by DR5-Fc (TRAIL-R2-Fc), whereas Fas-Fc was only slightly inhibitory and a control Fc had no effect (Figure 7B). Thus, TRAIL played a key role in T cell–mediated lysis of dendritic cells in this system. Next, we carried out direct ligand-dependent cell killing assays. We found that TRAIL induced significant dendritic cell death, whereas FasL and TNFα had no measurable effects (Figure 7C and data not shown). Moreover, dendritic cells from Pt 11 and Pt 36 underwent significantly less apoptosis in response to TRAIL compared to ALPS type I patients and healthy controls (Figure 7C). Apoptosis defects are also observed in Pt 11 mother and to a minor degree in the parents of Pt 36 (Figure 7C). These data suggest that the *CASP10* mutations in Pt 11 and Pt 36 affect TRAIL-induced death of dendritic cells.

To directly demonstrate that the defective CASP10

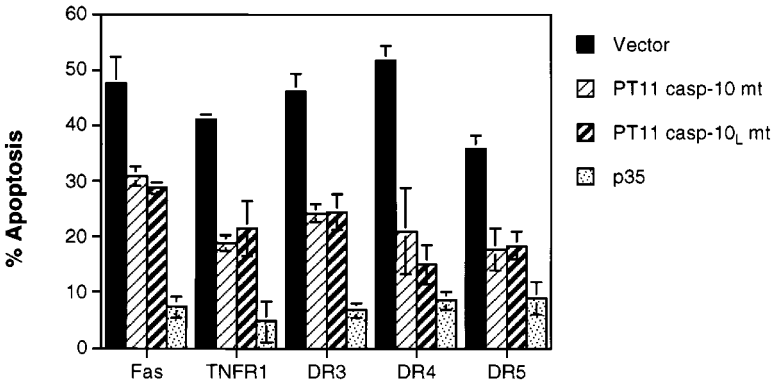


Figure 5. Dominant Interference with TNFR Family Member–Induced Apoptosis by Pt 11 CASP10

293 cells were cotransfected with Fas, TNFR1, DR3, DR4, or DR5 expression construct and a 4-fold excess of mutant CASP10, mutant $CASP10_L$, or a vector alone in the presence of a β-gal expression construct. Cells were stained, and transfected blue cells were quantitated 28 hr after transfection.

suppresses TRAIL-mediated apoptosis in dendritic cells, we used a retrovirus infection system to introduce the Pt 11 CASP10 mutant into dendritic cells. Two details of our procedure are noteworthy. First, we pseudotyped the Moloney-based vector with the vesicular stomatitis virus envelope glycoprotein (VSV-G) protein (Bartz and Vodicka, 1997), which dramatically improved the transduction of freshly cultured dendritic cells over that achievable with the Moloney envelope. Second, the Pt 11 *CASP10* gene was inserted upstream of an internal ribosome entry site (IRES) and the GFP gene, allowing us to monitor the efficiency of viral transduction and viral gene expression by flow cytometry. As shown in Figure 7E, >70% of the cells could be infected with either the retroviral construct carrying the CD4 gene (the control) or the vector encoding the Pt 11 mutant CASP10 (Figure 7D). When we compared the virally transduced dendritic cells to noninfected dendritic cells, we found that TRAIL-dependent killing was significantly reduced in cells bearing the Pt 11 CASP10-expressing virus (Figure 7D). By contrast, dendritic cells infected with CD4-expressing virus were killed as efficiently as uninfected cells by TRAIL. These data provide direct evidence that the Pt 11 mutant CASP10 dominantly interferes with TRAIL-mediated apoptosis in dendritic cells.

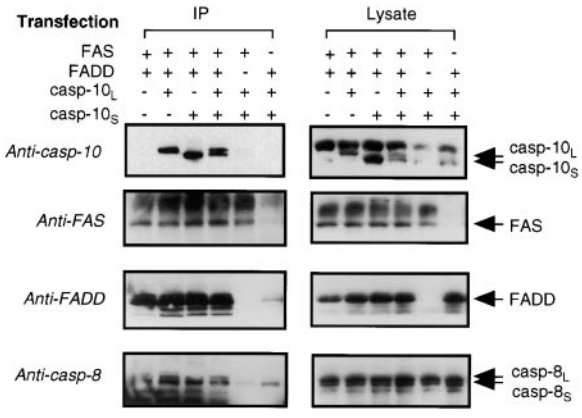


Figure 6. Recruitment of CASP10 to Fas

Human 293T cells were cotransfected with expression constructs for human FAS, FADD, and both isoforms of Pt 11 CASP10, as indicated. The transfected cells were harvested and lysed 20 hr later and immunoprecipitated with anti-APO-1 (anti-Fas) and sequentially probed with anti-CASP10, anti-FAS, anti-FADD, and anti-CASP8 as indicated (left). The lysates were also probed with the corresponding antibodies as shown (right).

To determine the physiological significance of the dendritic cell apoptosis defect, we compared frozen sections of lymph node from Pt 11 to lymph node tissues from ALPS type I patients and from individuals undergoing cancer staging purposes with no lymph node involvement. Both patient 11 and ALPS type I patients exhibited increased numbers of $CD3^+$ T cells in the parafollicular areas (Figure 7F). However, Pt 11 also exhibited unique large accumulations of CD83-bearing dendritic cells that were confined to the T cell areas of the lymph node. Dendritic cell accumulation was not observed in several ALPS type I and control lymph node specimens (Figure 7F and data not shown). Thus, abnormal dendritic cell apoptosis is associated with overaccumulation of dendritic cells in the lymph node in ALPS type II, which is not observed in ALPS type I.

## Discussion

Inherited mutations in Fas are currently the best known genetic link between apoptosis and the regulation of lymphocyte homeostasis and immune tolerance. Nonetheless, the broad range of clinical abnormalities of lymphoproliferation and autoimmunity in humans and mice harboring these mutations indicates that other genetic or environmental factors also influence these processes (Cohen and Eisenberg, 1991; Straus et al., 1999). Moreover, we and others have uncovered families that have ALPS-like disease but normal Fas and FasL genes (Dianzani et al., 1997; Sneller et al., 1997). The cellular and molecular bases of these abnormalities are poorly understood and have not been identified in the mouse models. The patients described in this report exhibit a range of diverse autoimmune and inflammatory conditions associated with disturbed immune cell homeostasis. Pt 11 is an important example in this regard. Since she was first evaluated in 1992, she has consistently exhibited dramatic lymphoproliferation and a breakdown in tolerance to multiple self-antigens leading to severe autoimmune complications. It was therefore surprising to find that she exhibited only a modest defect in Fas-induced apoptosis and no genetic abnormalities in Fas or FasL. We and others have found that disease spectrum and severity usually correlate with the degree of impairment of Fas-mediated apoptosis in ALPS type

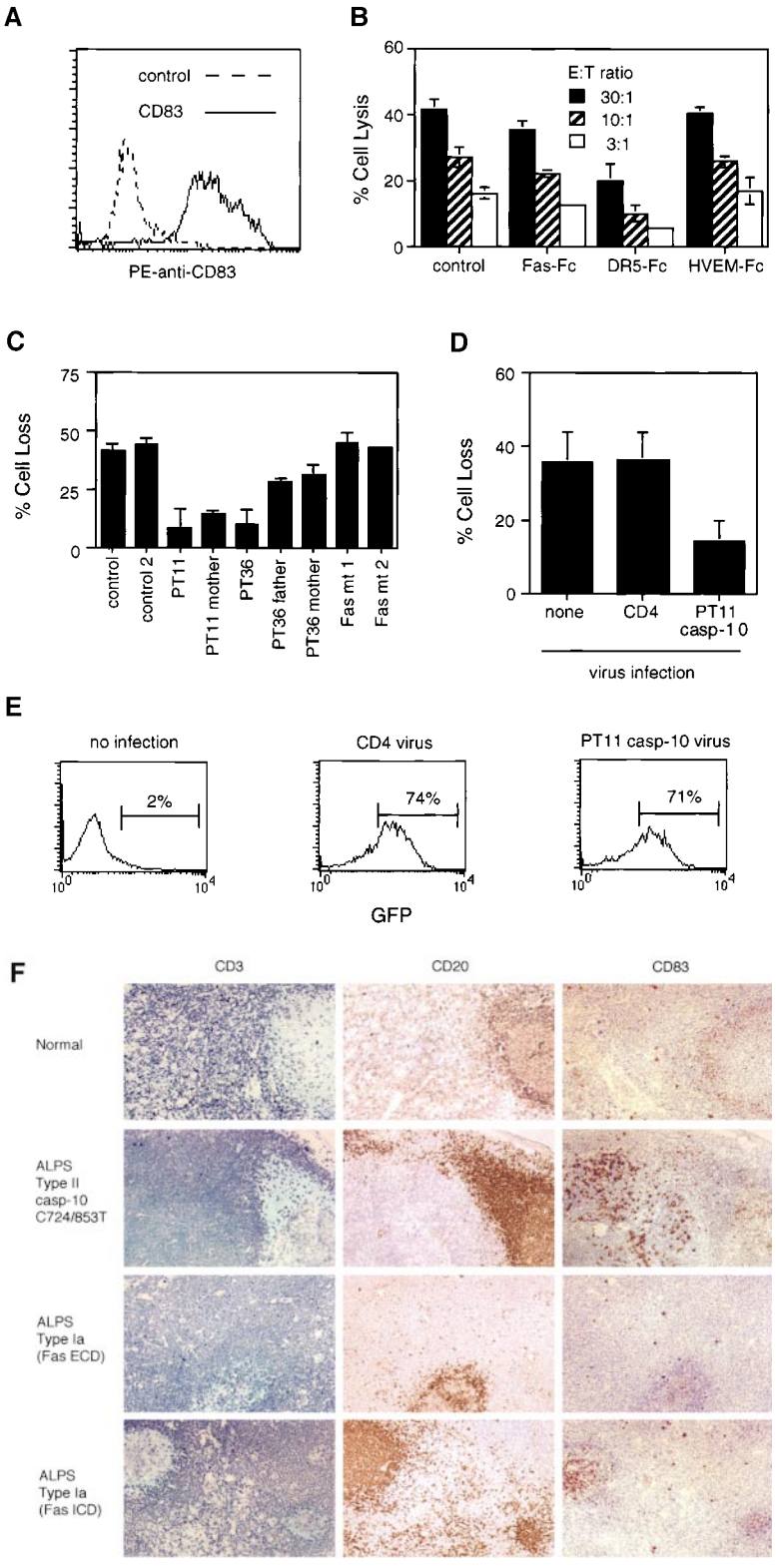


Figure 7. TRAIL-Mediated Killing of Dendritic Cells and Immunohistochemistry Staining of Dendritic Cells in Lymph Nodes

(A) Dendritic cells were incubated with PE-conjugated anti-CD83, and the example of one staining is shown.

(B) Lysis of dendritic cells by autologous $CD4^+$ T cells at various effector:target ratios in the presence of 10 μg/ml Fas-Fc, DR5-Fc (TRAIL-R2-Fc), or a control Fc protein.

(C) Dendritic cells from Pt 11 and Pt 36, family members, normal controls, and patients carrying Fas mutations were cultured with or without 1 μg/ml TRAIL at 37°C for 24 hr. Percent cell loss was measured as described previously.

(D) Dendritic cells infected with retrovirus expressing CD4 or Pt 11 CASP10 and uninfected dendritic cells were cultured with or without 1 μg/ml TRAIL at 37°C for 24 hr. Percent cell loss was measured as described previously.

(E) Dendritic cells with or without retroviral infection were analyzed by flow cytometry for GFP expression.

(F) Lymph node sections of Pt 11, two ALPS patients with Fas mutations, and a normal control were stained with CD83 for dendritic cells, CD3 for T cell areas, and CD20 for B cell areas.

I patients and their families (Jackson et al., 1999; Vaishnaw et al., 1999; R. Siegel et al., unpublished data). We can now account for the phenotype of the child and Pt 36 by the discovery of inherited mutations of the *CASP10* gene. In Pt 11, the allele is heterozygous, but the CASP10 protein encoded by her mutant allele is severely defective in autoprocessing and enzymatic function. The Pt 11 CASP10 can be recruited along with CASP8 into apoptosis signaling complexes and has a dominant-interfering effect on multiple TNFR-like death receptors,

including Fas, TNFR1, DR3, DR4, and DR5, in transfection assays. Pt 36 has a different mode of inheritance. The deficiency of his mutant *CASP10* is less severe, but homozygosity of this mutation results in a significant impairment of apoptosis. We found certain similarities to mice and humans with Fas defects such as the overaccumulation of lymphocytes, especially $CD4^{-}CD8^{-}$ T cells and autoimmune and inflammatory disorders. However, we also found an unprecedented defect in dendritic cell apoptosis in response to TRAIL and the overaccumulation of dendritic cells in the lymphoid tissue from Pt 11. The combined defect in lymphocyte and antigen-presenting cell apoptosis may have fundamental relevance to the pathogenesis of autoimmune disease in ALPS type II.

**CASP10 Connects to Important Apoptosis Pathways**

The physiological function of CASP10 is poorly understood. The gene was originally identified in the expressed sequence tag database, and transfection assays verified its function as a death-inducing caspase and indicated that it was recruited into apoptosis pathways especially involving DR4 and DR5 (Chaudhary et al., 1997; Pan et al., 1997; Schneider et al., 1997; Vincenz and Dixit, 1997). We found that the CASP10 mutations in Pt 11 and Pt 36 impair Fas-mediated apoptosis in lymphocytes and TRAIL-induced killing in both lymphocytes and dendritic cells. Moreover, transfection of the severely defective Pt 11 *CASP10* curtailed apoptosis induction by Fas, TNFR1, DR3, DR4, and DR5. This suggests that CASP10 affects a signal integration point for apoptosis receptors. The downstream position of its effect at a point common to multiple cell death pathways could account for the severe pleiotropic autoimmunity and inflammatory disorders and abnormal cellular homeostasis in these two patients with ALPS type II.

The signaling role of CASP10 vis-à-vis its close homolog CASP8 is not understood. The *CASP8* and *CASP10* genes appear to be part of a gene duplication event in the human *CASP8/10/c-FLIP* locus (2q33, interval 204.7–205.4). It is now evident that CASP10 in its mutant form impairs apoptosis in the presence of a normal *CASP8* gene. Genetic deficiencies of *CASP8* have been shown to block Fas- and TNF-induced apoptosis (Juo et al., 1998; Varfolomeev et al., 1998). Thus, CASP10 is unlikely to be a redundant form of CASP8. The third homolog in the locus, c-FLIP, contains multiple amino acid substitutions that abrogate its enzymatic function and can either impair or enhance apoptosis by associating with apoptosis signaling proteins in transfected cells (Goltsev et al., 1997; Irmler et al., 1997; Shu et al., 1997; Srinivasula et al., 1997; Rasper et al., 1998). In a manner similar to c-FLIP, the mutant CASP10 could interfere with CASP8 activation. Our data show that CASP8 and CASP10 can be corecruited by FADD to Fas (Figure 6), but the relative contribution of either caspase to death induction by each of the death receptors is not defined. In family 36, the milder form of mutant CASP10 did not alone cause a strong dominant-interfering effect as evidenced by the minor apoptosis defect in each parent who was heterozygous and clinically normal. This could imply that the severity of apoptosis defect may be correlated with clinical phenotype independent of any other genetic or environmental influences. Dominant-negative as well as homozygous recessive mutants for *CASP10* may be important tools to dissect these pathways in various immune cell types. A prime example may be dendritic cells, for which little is known about the pathways that govern their fate during and after immune responses.

**Mutation Affects Autoprocessing and Biochemical Function**

The inherited mutations of CASP10 that we found are both in the p17 subunit. The mutant allele in Pt 11 leads to a substitution of leucine for phenylalanine at position 242. In Pt 36, a substitution of isoleucine for valine occurs at position 367. The p17 subunit contains the active site of the enzyme, and both mutants show impaired caspase function. Although the crystal structure of CASP10 has not been solved, the conservation of key amino acids allowed us to make a plausible model of the CASP10 p17 based on the structures of CASP1 and CASP3 (R. Siegel and M. J. L., unpublished results; Walker et al., 1994; Rotonda et al., 1996). For Pt 36, the mutation alters a valine located seven amino acids downstream of the active-site QACQG motif in a region that is not structurally conserved in CASP3 and CASP1 (Rotonda et al., 1996). This amino acid change may have its functional effect by virtue of its proximity to the catalytic site rather than causing a global change in the p17 structure. For Pt 11, the model suggests that Leu-242 is equivalent to Leu-164 in the first β strand of CASP3. This position is not near the substrate-binding pocket but at an internal location of the p17 subunit. The relatively conservative nature of substitution of Phe for Leu (i.e., both are hydrophobic) and the fact that enzymatic activity is not completely abolished make it unlikely that the mutant p17 subunit is completely unfolded. Also, Leu-242 is not sufficiently close to the interface of the large and small subunits or the aspartates that are cleaved during maturational processing to suggest that it has a direct effect on processing or assembly. Rather, we favor the notion that there is a local perturbation of the β strand containing Leu-242. This strand directly connects to the P1 loop that contains Arg-179, which plays a crucial role in catalysis by stabilizing the P1 aspartic acid of the substrate, suggesting that the defect in autoprocessing appears to be secondary to defective enzymatic function. Further evidence that the Pt 11 mutant p17 folds in an essentially correct manner is the fact that weak dominant-interfering effects can be obtained by expressing the mutant subunit alone (J. W. and M. J. L., unpublished results). Thus, the dominant interference effect results from a subtle structural change that impairs catalytic activity, which apparently prevents autoprocessing after recruitment into apoptosis signaling complexes.

**Dendritic Cell Homeostasis by Apoptosis May Be Crucial for Immune Tolerance**

Previous investigations of autoimmunity and apoptosis have focused almost exclusively on lymphocytes. It is now evident that the turnover of antigen-presenting cells is an important factor in the maintenance of tolerance. The differentiation and growth of dendritic cells have been well studied, but little is known about their fate

after migration to the lymphoid organs (Banchereau and Steinman, 1998). The TNF family molecules CD40 ligand and TRANCE produced by T cells can promote the survival of dendritic cells in lymphoid tissue (Anderson et al., 1997; Wong et al., 1997). However, Jenkins and coworkers have observed that dendritic cells are directly eliminated in an antigen- and T cell–dependent manner soon after an immune reaction (Ingulli et al., 1997). We now show that activated T cells directly induced the death of dendritic cells. Moreover, of the three death-inducing cytokines produced by antigen-stimulated T cells that were tested, TRAIL, but not FasL or TNF, had the strongest apoptosis-inducing effect on dendritic cells. Moreover, *CASP10* mutations impaired TRAIL-induced dendritic cell apoptosis. Corresponding to this apoptosis defect, we found a marked accumulation of dendritic cells in the T cell areas of the lymph nodes in Pt 11. Taken together with the findings of Ingulli et al. (1997) and the fact that TRAIL is produced by T cells (as well as B cells and monocytes) (Marsters et al., 1996; Thomas and Hersey, 1998; Griffith et al., 1999), the physiological disappearance of dendritic cells could be mediated by TRAIL. Similar lines of evidence indicate that this regulatory pathway is unimpaired by Fas mutations in ALPS type I.

Dendritic cell persistence could cause prolonged stimulation of T cells and B cells (Banchereau and Steinman, 1998). Usually, repeated antigen stimulation of activated T cells leads to apoptosis by the propriocidal mechanism (Lenardo, 1996). Our data suggest that there is a cross-regulation in which TRAIL produced by T cells can induce dendritic cell apoptosis. However, in ALPS type II, both T cells and dendritic cells have impaired apoptosis responses and accumulate in T zones where the lingering dendritic cells could induce responses that do not normally take place. The combined apoptosis defects in two interacting cell types (i.e., dendritic cells and T cells) may be responsible for the disease phenotype in ALPS type II.

## Both Dominant and Recessive Mutations May Produce Disease Due to Apoptosis Dysfunction

Pt 11 and Pt 36 have been developmentally normal thus far and manifested disease primarily in the postnatal period. Pt 36 has inherited two identical defective alleles from unaffected heterozygous parents in the manner of a classic Mendelian recessive trait. The Pt 36 *CASP10* mutation is a G to A mutation that occurs at a CpG dinucleotide, which is a known "hot spot" for C to T transitions involved in human diseases (Kotze et al., 1997). An increased allele frequency in the Ashkenazi Jewish population was ruled out in our screening. Homozygosity in Pt 36 may reflect common ancestry of mother 36 and father 36, though they are unaware of any consanguinity.

The fact that Pt 11 had severe disease with a mutation that was heterozygous and strongly dominant interfering is significant. The results of experiments with null defects for individual caspases in mice reveal either surprisingly little apoptosis phenotype or perinatal lethality (Li et al., 1995; Kuida et al., 1996, 1998; Ghayur et al., 1997; Hakem et al., 1998; Varfolomeev et al., 1998; Wang et al., 1998). Although homozygous loss-of-function mutations have been rarely observed, most ALPS type I patients have Fas gene mutations that are heterozygous and dominant interfering (Fisher et al., 1995; Rieux-Laucat et al., 1995; Drappa et al., 1996; Bettinardi et al., 1997; Kasahara et al., 1998). Evidence that the heterozygous expression of the Pt 11 mutant CASP10 is sufficient for immune pathology is provided by her mother who has the same heterozygous genotype and manifests high titers of autoantibodies and apoptosis defects. Dominant interference in ALPS reflects the fact that receptor-mediated apoptosis requires multimeric protein–protein signaling complexes that are vulnerable to the interposition of defective proteins. Although CASP10 is expressed in many tissues, it is abundant in hematopoietic cells, which could explain why Pt 11 and Pt 36 have chiefly immunological defects (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997). Dominant-interfering mutations of other caspases could impair selected apoptosis pathways and thereby manifest themselves as disease predominantly affecting other organ systems.

## Experimental Procedures

### Detection of Patient Mutations

RT–PCR and DNA sequencing were performed as described (Fisher et al., 1995). Amplification primers for *CASP10* cDNA were as follows: forward primer, 5′-GAGAACCGTTTACTTCCAGAAGATTG-3′; reverse primer, 5′-GCTGGAGGTTATACCCAATGATTCG-3′. The *CASP10* clones were sequenced, and clones free of errors were used for experiments. Amplification primers for genomic sequences surrounding the patient 11 *CASP10* mutation site were as follows: forward primer, 5′-CAGCTGTGTACAGGATGAATCGG-3′; reverse primer, 5′-CATCTTTATGGGTTCCTTGTCTGTC-3′. A 76 bp product amplified from genomic DNA was digested with Stu I followed by electrophoresis on 3% agarose gels. Amplification primers for genomic sequences surrounding the patient 36 *CASP10* mutation site were as follows: forward primer, 5′-ACAGCCCTGCAGTGCCCT AGA-3′; reverse primer, 5′-ACTGTCCTGCAGGGAAGTGGG-3′. The frequency of patient 36 *CASP10* mutation was analyzed by SSCP analysis of amplified genomic DNA samples.

### Cell Culture and Apoptosis Assay

Human PBLs were stimulated with PHA and then IL-2 as described (Fisher et al., 1995). Dendritic cells were cultured as described previously (Sallusto and Lanzavecchia, 1994). Apoptosis induced by anti-Fas (CH11) and anti-CD3 (64.1) were analyzed as described (Fisher et al., 1995). A soluble TRAIL tagged at the N terminus with a leucine zipper motif is a generous gift from Dr. David Lynch at Immunex (Walczak et al., 1999). To analyze TRAIL-mediated apoptosis, T cells or dendritic cells were incubated in culture medium with or without 1 μg/ml TRAIL at 37°C for 24 hr, and cell loss induced by TRAIL was calculated as described (Fisher et al., 1995).

### Transfection

MCF7, HeLa, and 293 cells were transfected with various constructs plus pcDNA3-LacZ by the Superfect method (Qiagen). Twenty-four hours after transfection, the cells were fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 5 min. The cells were then stained by incubation in PBS containing 1 μg/ml X-gal, 5 mM ferricyanide, 5 mM ferrocyanide, 2 mM $MgCl_2$, 0.02% NP-40, and 0.01% SDS at 37°C. Live and dead cells stained with blue color were scored under a microscope.

### Expression of GST-CASP10 and Protease Assay

The CASP10 protease domain was cloned into pGEX-4T.1 and expressed in BL21plyss, and the GST-CASP10 fusion protein was induced by 0.1 mM IPTG. Bacteria from a 30 ml culture were resuspended in 500 μl cell extraction buffer (25 mM HEPES [pH 7.5], 0.1% CHAPS, 5 mM EDTA, and 2 mM DTT) and lysed by freezing

and thawing, followed by sonication. The fusion proteins from 50 μl bacterial lysates were purified by glutathione Sepharose (Pharmacia) and used for SDS–PAGE or protease assay. To assay protease activity, CASP10 extracts were added to 200 μl protease buffer (30 mM HEPES [pH 7.4], 10% sucrose, 1 mM $CaCl_2$, 5 mM DTT) containing 50 μM Asp-Glu-Val-Asp-AMC (DEVD-AMC) or 50 μM Tyr-Val-Ala-Asp-MCA (YVAD-MCA) and incubated at room temperature. The released MCA were measured by excitation at 390 nM and absorbance at 460 nM in a fluorospectrophotometer (Tecan). Bacterial GST-CASP10 fusion proteins were purified on glutathione agarose beads and eluted with 5 mM glutathione. The fusion proteins were quantitated using Coomassie blue method (Biorad), and 2 μg of each fusion protein was used for 200 μl caspase reactions. For $K_m$ determination, varying amounts of DEVD-AMC were used, and the initial velocities of the reaction were determined when less than 5% of the substrate has been converted, generally within the first 5–10 min of the reaction. The $K_m$ value was calculated by using the Lineweaver-Burk equation.

### Transfection of 293T Cells and Fas, FADD, and CASP10 Association

Human 293T cells were cultured to 50%–80% confluency in six-well plates. The cells were then cotransfected with 0.75 μg of each expression construct for human FAS, FADD, and long or short isoforms of Pt 11 CASP10 using the Fugene transfection reagent (Boehringer Mannheim) and cultured for 20 hr. The cells were lysed on ice for 10 min with 400 μl lysis buffer containing 30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, and 1× protease inhibitor cocktail (Boehringer Mannheim), and the supernatants were used for immunoprecipitation and Western blotting analysis. To determine Fas/FADD/CASP10 association, 1 μg/ml anti-APO-1 (anti-Fas; Kamiya) was added to 350 μl lysate on ice for 30 min. The FAS-associated molecules were coimmunoprecipitated at 4°C with 20 μl protein G beads for 2 hr with rotation. The beads were washed five times with lysis buffer, analyzed by SDS–PAGE, transferred to nitrocellulose membranes, probed with affinity-purified rabbit anti-human CASP10 (gift from Dr. Donald Nicholson, Merck), and developed by chemiluminescence (Pierce). For sequential reprobing, the membranes were first stripped with the stripping buffer (65 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM 2 mercaptoethanol) at 68°C for 15 min, then probed as described above with anti-FAS, anti-FADD, or anti-CASP8 antibodies.

### Retroviral Infection

The retroviral cloning vector rKat43.267F3-CD4-IRES.GFP and packaging vector pKat2.Ampac.UTd were general gifts from Dr. Margo Roberts (Finer et al., 1994). Pt 11 mutant CASP10 was cloned into the retroviral cloning vector by replacing the CD4 gene before the IRES-GFP sequence. For retrovirus production, the retroviral cloning and packaging vectors were cotransfected into 293T cells essentially as described (Finer et al., 1994), and a CMV-VSV-G expression plasmid was included to pseudotype the retroviral vector with VSV-G for improved infection of target cells (Bartz and Vodicka, 1997). Supernatants of transfected 293T cells were filtered through a 0.45 μM filter and used for spin infections of dendritic cells in the presence of 4 μg/ml polybrene (Sigma) over a 24 hr period. Infections with either the CD4 or patient 11 CASP10 viruses were monitored by GFP expression by flow cytometry and used for TRAIL killing assays.

### T Cell–Mediated Lysis of Dendritic Cells

To purified $CD4^+$ T cells, T cells stimulated by PHA and IL-2 were incubated with FITC-anti-CD8 (Pharmingen) and anti-FITC-conjugated magnetic beads (PerSeptive Diagnostics). Following removal of cells bound to the beads, the remaining cells are >98% for CD4 staining (data not shown). For T cell–dependent assays, dendritic cells ($10^4$/well) labeled with $Na[^{51}Cr]O_4$ (Amersham) were mixed with autologous $CD4^+$ T cells at different effector:target ratios in 96-well round-bottomed plates in RPMI complete medium (200 μl/well) containing 5 μg/ml 64.1 (anti-CD3) with or without 10 μg/ml Fas-Fc, DR5-Fc (TRAIL-R2-Fc; Alexis), or control Fc (HVEM-Fc; Montgomery et al., 1994) as indicated. After incubation at 37°C for 8 hr, supernatants were harvested and measured in a γ counter, and percentage cell lysis was quantitated as described (Wang and Lenardo, 1997).

### Immunohistochemistry Staining

Sections of frozen lymph node tissues were stained with anti-CD83 (Immunotech), anti-CD3 (Becton Dickinson), or anti-CD20 (Dako) followed by biotinylated secondary antibody and HRP-conjugated streptavidin (Vector). The sections were then developed with the substrate kit containing DAB/metal and peroxidase substrate kit (Pierce) for CD20 and CD83 staining or the ABC immunoperoxidase technique (Lim et al., 1998) for CD3 staining. The cell nuclei were counterstained with hematoxylin.

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