

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

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Abstract Fas stimulation has been reported to promote the activation and proliferation of T lymphocytes, but the intracellular signalling pathways that mediate non-apoptotic responses to Fas are poorly defined. To distinguish between the activation signalling and the death-inducing pathway downstream of Fas, we generated a novel T cell line expressing a chimeric hCD8-FasC protein and found that stimulation with the anti-CD8 antibodies induced tyrosine phosphorylation of TCR-proximal proteins, activation of Raf-1/ERK, p38 and JNK, and increased expression of CD69, Fas, and Fas ligand. Stimulation of hCD8-FasC-induced activation of an atypical NF-κB pathway, partial cleavage of caspases, and increased expression of TRAF1, FLIP_L and FLIP_S, thereby protecting T cells from FasL-mediated apoptosis. The proliferative response transmitted through hCD8-FasC chimeric receptors was converted into

death signals when cells were stimulated, resulting in increased expression of IL-2 and Nur77 and increased caspase cleavage. Surprisingly, both the enhanced expression of FLIP_L and FLIP_S and the complete inhibition of FLIP_S expression were functionally associated with cell death induction. These findings imply that Fas is able to trigger intracellular signalling events driving both apoptosis and activation of T cells but that cell fate is determined by quantitative and qualitative differences in intracellular signalling following Fas stimulation.

Keywords Fas · Apoptosis · Activation signals · T cells

1 Introduction

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

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

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

To investigate the molecular nature of non-apoptotic signalling events induced by Fas, we generated stable Jurkat T cell transfecants expressing an hCD8-FasC fusion protein on the cell surface to enable the differentiation of Fas-mediated activation signals from cell death-inducing signals. We found that activation of the hCD8-FasC chimeric receptor by the anti-CD8 mAbs UCHT4 and OKT8

not only induced early and late activation signals leading to cell proliferation but also activated an atypical NF- κ B pathway that inhibited FasL-induced cell death. Moreover, an increase in intracellular Ca²⁺ or PKC activation converted activation signals through the hCD8-FasC chimeric receptor into death signals by enhancing IL-2 and Nur77 expression. In contrast to previous results demonstrating a concentration-dependent anti-apoptotic function of c-FLIP, our data suggest that two independent signalling pathways in which the expression of both FLIP_L and FLIP_S is increased by phorbol 12-myristate 13-acetate (PMA) or FLIP_S expression is inhibited by ionomycin (IM) may play an important role in the conversion of Fas-mediated activation to cell death. These results provide the first molecular evidence that qualitative and quantitative differences in signalling through the Fas receptor can modulate the fate of T cells, driving them toward either cell death or activation, and our study may have clinical benefits to the Fas-associated dysfunction such as self reactivity, immune dysfunction, malignant transformation [19].

2 Materials and methods

2.1 Construction of CD8 chimeric receptors

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

2.2 Antibodies and reagents

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

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

2.3 Transfection

For stable transfection, Jurkat cells (1×10^7) were washed with PBS supplemented with 0.1 M MgCl₂ and incubated with 25–50 μ g plasmid on ice for 10 min and then electroporated using an ECM 600 electroporator (BTX Inc. Holliston, MA) at 1.2 kV and 25 μ F. After pulsing, the cells were incubated in T75 culture flasks for 48 h at 37°C and plated in 96-well plates with medium containing 2.2 mg/ml geneticin (Gibco BRL, Rockville, MD). 293T cells were transiently transfected with 1 μ g plasmid using LipofectAMINE_{TM} Reagent (Gibco BRL, Rockville, MD) according to the manufacturer's instructions.

2.4 RT-PCR analysis and RPA assay

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

2.5 Apoptosis analyses

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

2.6 Immunoprecipitation and western blot analysis

After incubation of 3×10^7 hCD8-FasC transfectants with the cross-linked OKT8 and UCHT4 antibodies (1 μ g/1 $\times 10^6$ cells) at 37°C, cells were lysed in lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 mM Na₃VO₄, 200 mM PMSF, 10 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 1% NP-40). Tyrosine-phosphorylated proteins were immunoprecipitated using anti-p-Tyr antibody coupled to agarose beads and washed in 1:5-diluted lysis buffer. Immunoprecipitates or total cell lysates were fractionated by SDS-PAGE and transferred to Immunobilon-P (Millipore, Bedford, MA) membranes. Membranes were incubated with primary antibodies, washed, incubated with horseradish peroxidase-conjugated secondary antibodies, and developed with ECL (Amersham, Uppsala, Sweden).

3 Results

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

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

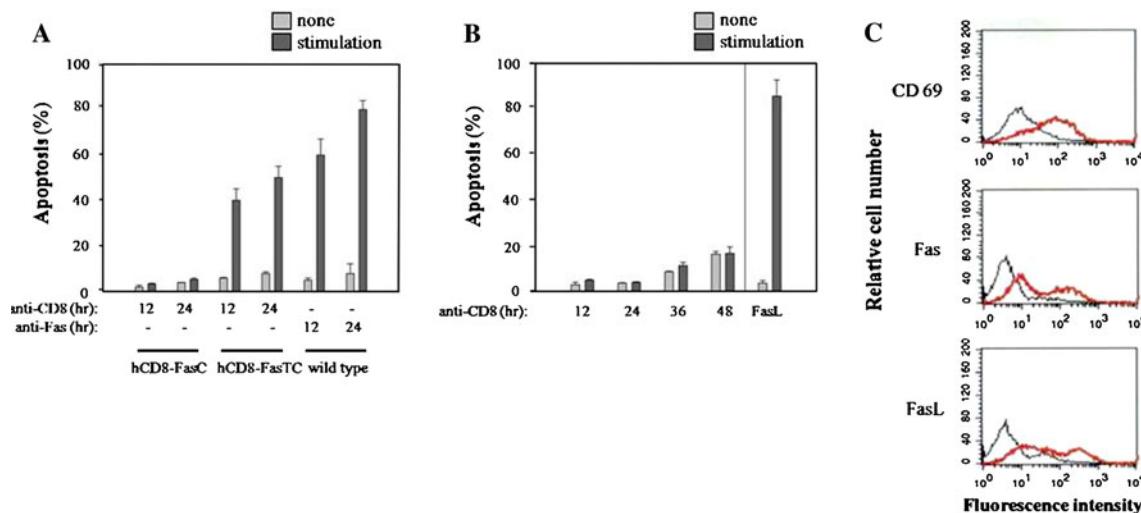


Fig. 1 Cell death was not induced by stimulation of hCD8-FasC chimeric receptor. **a** 293T cells transiently transfected with the hCD8-FasC DNA construct were stimulated with immobilised OKT8 and UCHT4 antibodies ($1 \mu\text{g}/10^6$ cells) for 12 or 24 h, and cell death was analysed. As a control, cells were stimulated with immobilised anti-Fas antibodies. **b** Stable Jurkat T cell transfectants expressing the hCD8-FasC chimeric receptor were stimulated for the indicated

periods with immobilised OKT8 and UCHT4 or with NIH3T3 cells stably expressing FasL, and cell death was analysed by PI exclusion. **c** hCD8-FasC Jurkat transfectants were stimulated for 8 h with immobilised OKT8 and UCHT4 antibodies (red lines). Surface expression levels of CD69, Fas and FasL were analysed by flow cytometry

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

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

To examine whether the tyrosine phosphorylation of various intracellular signalling proteins, a hallmark of T cell activation, can be induced by hCD8-FasC stimulation,

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

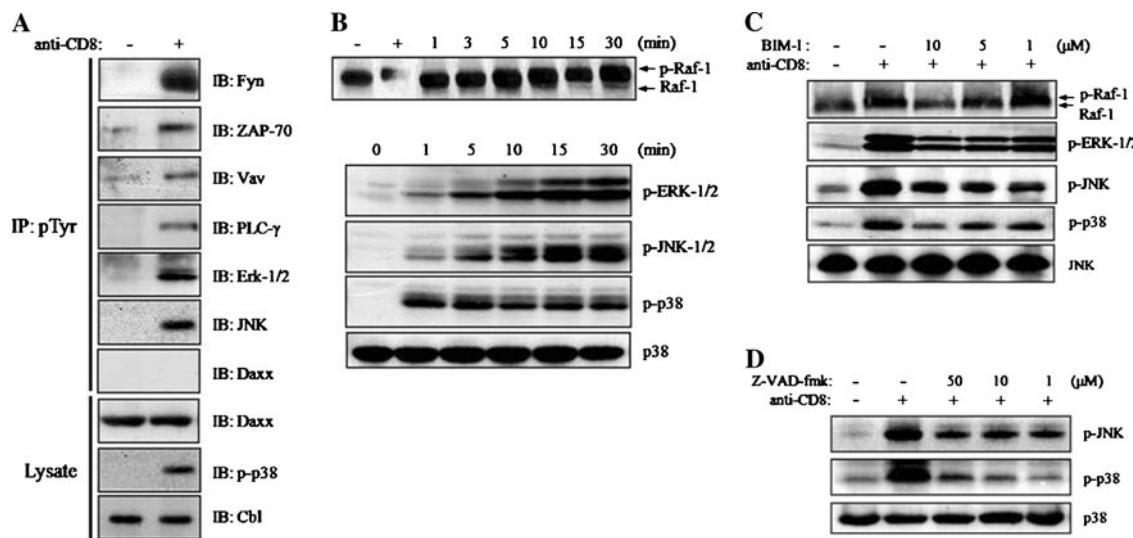


Fig. 2 hCD8-FasC-mediated signalling activates multiple intracellular proteins involved in T cell activation. **a** hCD8-FasC Jurkat transfectants were stimulated for 10 min with or without cross-linked OKT8 and UCHT4 antibodies. Cell lysates were immunoprecipitated with anti-pTyr Ab-conjugated agarose beads and immunoblotted with the indicated antibodies. **b** hCD8-FasC Jurkat transfectants were stimulated for the indicated periods with cross-linked OKT8 and

UCHT4 antibodies, and cell lysates were immunoblotted with antibodies specific to phospho-Erk1/2, phospho-JNK or phospho-p38. hCD8-FasC Jurkat transfectants were treated for 1 h with or without BIM-I (1, 5, or 10 μ M) (**c**) or Z-VAD-fmk (1, 10, or 50 μ M) (**d**) and stimulated with OKT8 and UCHT4 antibodies for 10 min. Cell lysates were immunoblotted with antibodies specific to phospho-Erk1/2, phospho-JNK or phospho-p38

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

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

Previous reports have implicated the NF- κ B pathway in Fas-mediated cell survival; however, these findings remain controversial [15]. To determine whether hCD8-FasC-mediated activation signalling induces the activation of NF- κ B, hCD8-FasC transfectants were stimulated with UCHT4 and OKT8, lysed, and immunoblotted with anti-phospho-I κ B- α antibody. Activation of the T cells through

hCD8-FasC immediately induced phosphorylation of I κ B- α (Fig. 3a), and this hCD8-FasC-mediated NF- κ B activation was dependent on caspase activity, but not on the canonical PKC-mediated NF- κ B activation pathway (Fig. 3b). We next performed an RPA assay to identify anti-apoptotic proteins induced by hCD8-FasC-mediated activation (Fig. 3c). Stimulation of hCD8-FasC induced a significant increase in expression of TRAF1, but not TRAF2, and this increase in TRAF1 expression was completely inhibited by the NF- κ B inhibitor sodium salicylate (Na-Sal).

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

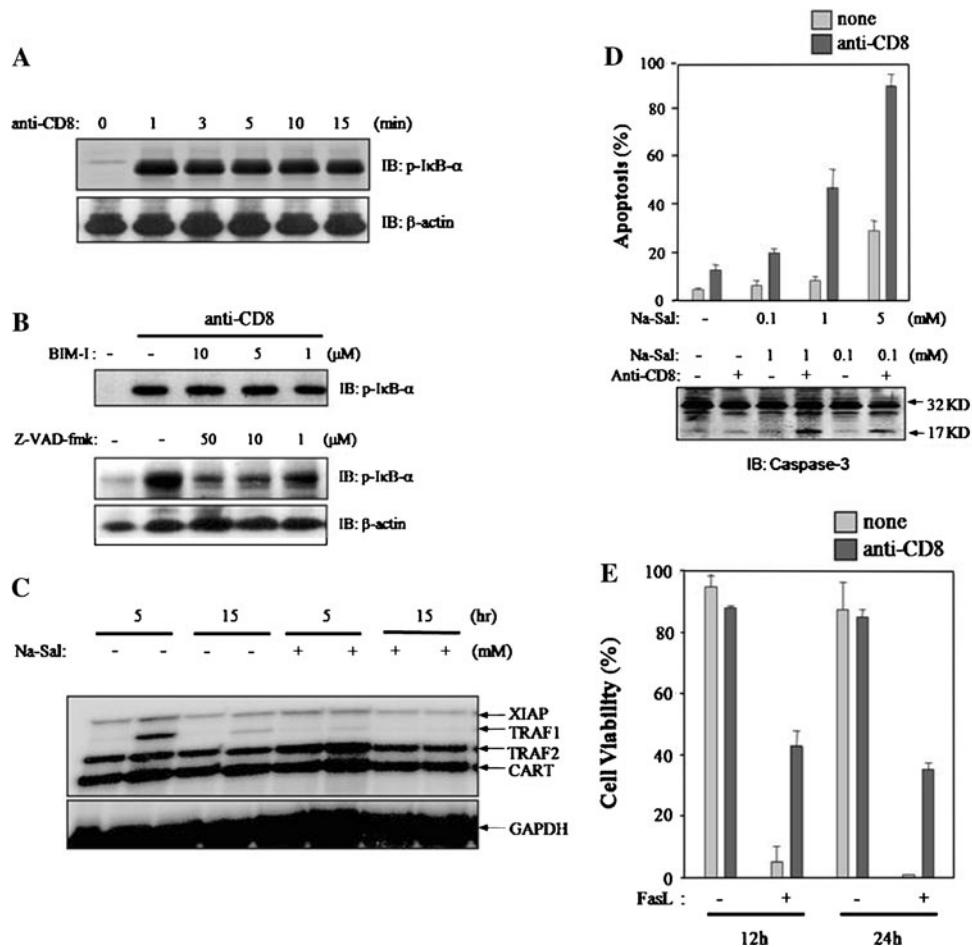


Fig. 3 hCD8-FasC-mediated NF- κ B activation inhibits Fas-mediated apoptosis. **a** hCD8-FasC Jurkat transfectants were stimulated with cross-linked OKT8 and UCHT4 antibodies for the indicated periods, and cell lysates were immunoblotted with anti-phospho-I κ B- α antibodies. **b** hCD8-FasC Jurkat transfectants were treated for 1 h with or without various concentrations of BIM-I (1, 5, or 10 μ M) or Z-VAD-fmk (1, 10, or 50 μ M) and stimulated with cross-linked OKT8 and UCHT4 antibodies for 10 min. NF- κ B activation was detected by anti-phospho-I κ B- α antibodies. **c** hCD8-FasC Jurkat transfectants were stimulated for 5 or 15 h with immobilised OKT8 and UCHT4 antibodies. Cell lysates were immunoblotted with antibodies specific for XIAP, TRAF1, TRAF2, CART, and GAPDH.

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

3.4 Elevated PKC activation or intracellular Ca²⁺ convert hCD8-FasC-mediated activation signals into death signals

We next investigated whether hCD8-FasC-mediated T cell activation in combination with strong T cell signals such as elevated PKC activation or Ca²⁺ influx results in cell death. Treatment with PMA or IM induced a significant increase in cell death in hCD8-FasC transfectants stimulated through hCD8-FasC, as measured by DNA fragmentation (Fig. 4a). Induction of apoptosis upon stimulation through hCD8-FasC

and UCHT4 antibodies in the absence or presence of 5 mM sodium salicylate. Induction of mRNAs encoding anti-apoptotic proteins was analysed by RPA. **d** hCD8-FasC Jurkat transfectants were stimulated for 12 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of sodium salicylate. Cell death was analysed by PI staining. The cleavage of caspase-3 was analysed by immunoblotting with anti-caspase-3 antibodies. **e** After hCD8-FasC Jurkat transfectants were stimulated for 12 h or 24 h with or without immobilised OKT8 and UCHT4 antibodies, cells were incubated with NIH3T3-FasL for 90 min. Cell death was analysed by PI staining

in the presence of PMA or IM was significantly blocked by cyclosporine A (CsA) or BIM-I, respectively, suggesting that the low levels of both PKC activation and Ca²⁺ influx induced by hCD8-FasC stimulation are required for hCD8-FasC-mediated apoptosis. Similar results were obtained in 293T cells transiently expressing hCD8-FasC (Fig. 4b). To confirm the effect of PKC activation or Ca²⁺ influx on Fas-induced cell death, we stimulated primary mouse T cells with two immobilised anti-Fas antibodies, which are known to induce apoptosis slowly, in the absence or presence of PMA or IM. Treatment with PMA or IM significantly enhanced Fas-mediated cell death in primary T cells (Fig. 4c). It has been demonstrated that caspase activation is required for induction of Fas-mediated apoptosis in Jurkat T

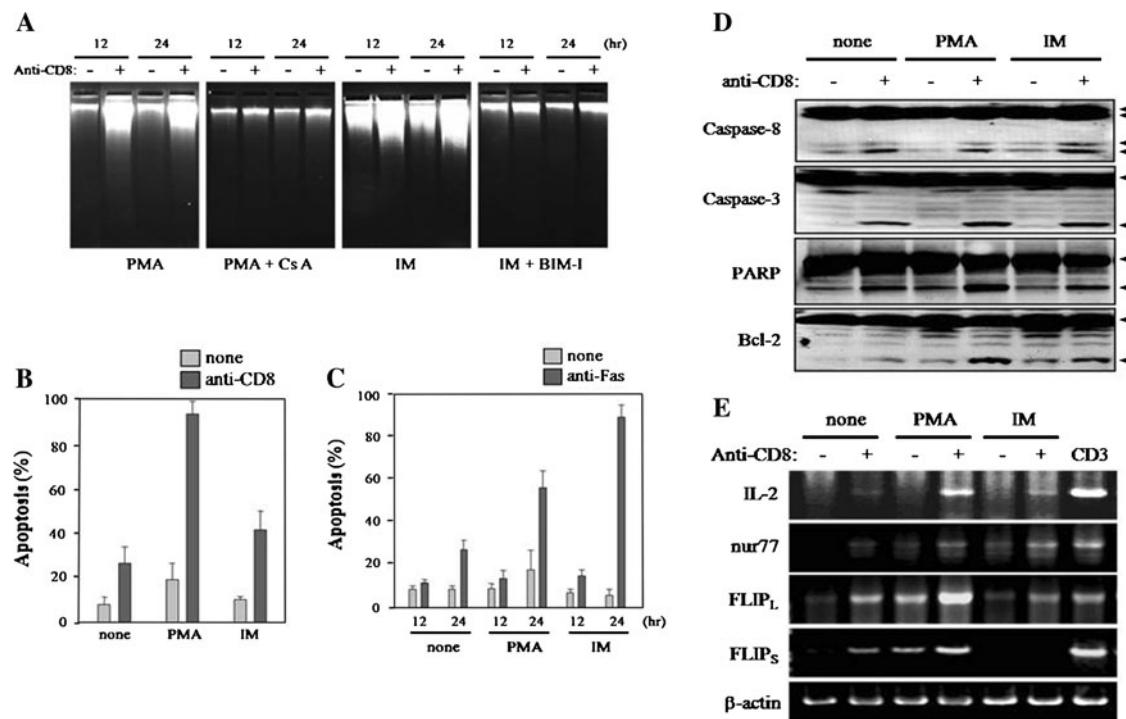


Fig. 4 PMA or ionomycin convert hCD8-FasC-mediated activation signals to death signals. **a** hCD8-FasC Jurkat transfectants were stimulated for 12 h or 24 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA, 1 ng/ml PMA and 10 µg/ml cyclosporine A, 50 nM IM, or 50 nM IM and 1 µM BIM-I. Cell death was analysed by DNA fragmentation assay. **b** 293T cells were transiently transfected with hCD8-FasC DNA and stimulated with cross-linked OKT8 and UCHT4 antibodies in the absence or presence of PMA or IM. Cell death was analysed by PI exclusion. **c** Peripheral T cells were stimulated for 12 or 24 h with two immobilised agonistic anti-Fas antibodies, ZB4 and G254-274, in the absence or presence of 5 ng/ml PMA or 100 nM IM. Cell death was

analysed by PI exclusion. **d** hCD8-FasC Jurkat transfectants were stimulated for 1 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA or 50 nM IM, and cell lysates were immunoblotted with the indicated antibodies. **e** hCD8-FasC Jurkat transfectants were stimulated for 5 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA or 50 nM IM. RNA was extracted from the cells, and RT-PCR analysis was performed using primers specific for IL-2, Nur77, FLIP_L and FLIP_S. As a positive control, cells were stimulated with immobilised OKT3 antibodies in the presence of 1 ng/ml PMA or 25 ng PMA and 200 nM IM

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

Stimulation of the TCR complex has been reported to induce sensitivity to cell death by increasing the expression of Fas, FasL, IL-2, and Nur77 in T cell hybridomas [23]. To determine whether hCD8-FasC-mediated apoptosis induction in the presence of PMA or IM results in increased IL-2 and Nur77 expression, hCD8-FasC transfectants were stimulated with immobilised UCHT4 and OKT8 in combination with PMA or IM, and expression of IL-2, Nur77, FLIP_L and FLIP_S was analysed (Fig. 4e).

Although hCD8-FasC stimulation resulted in only a slight induction of Nur77 expression, both PMA and IM significantly increased Nur77 mRNA expression levels. The levels of Nur77 mRNAs induced by hCD8-FasC stimulation in combination with PMA or IM were comparable to those observed upon TCR stimulation. Stimulation of hCD8-FasC induced a substantial increase in IL-2, FLIP_L and FLIP_S expression that was further enhanced only by PMA; IM treatment resulted in a significant abrogation of hCD8-FasC-mediated FLIP_S induction. In contrast, hCD8-FasC stimulation in the presence of either PMA or IM effectively induced cell death. The level of IL-2 induction by hCD8-FasC was not increased by IM, suggesting that the signalling pathway leading to cell death downstream of TCR stimulation may be similar to that downstream of hCD8-FasC and PKC activation but different from that downstream of hCD8-FasC and increased Ca²⁺ influx. Taken together, these results demonstrate that enhanced PKC activation or an increase in intracellular Ca²⁺ influx

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

4 Discussion

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

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

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

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

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

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

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