CD95 death-inducing signaling complex formation and internalization occur in lipid rafts of type I and type II cells

Adriana Eramo[1], Massimo Sargiacomo[1], Lucia Ricci-Vitiani[1], Matilde Todaro[2], Giorgio Stassi[2], Carlo G. M. Messina[1], Isabella Parolini[1], Fiorenza Lotti[1], Giovanni Sette[1], Cesare Peschle[1,3] and Ruggero De Maria[1,4]

[1] Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy
[2] Department of Surgical and Oncological Sciences, University of Palermo, Palermo, Italy
[3] Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, USA
[4] Department of Experimental Oncology, Istituto Oncologico del Mediterraneo, Catania, Italy

We investigated the membrane localization of CD95 in type I and type II cells, which differ in their ability to recruit and activate caspase-8. We found that CD95 was preferentially located in lipid rafts of type I cells, while it was present both in raft and non-raft plasma membrane sub-domains of type II cells. After stimulation, CD95 located in phospholipid-rich plasma membrane was recruited to lipid rafts in both types of cells. Similarly, CD95 cross-linking resulted in caspase-independent translocation of FADD/MORT1 and caspase-8 to the lipid rafts, which was prevented by a death domain-defective receptor. CD95 internalization was then rapid in type I and delayed in type II cells and showed a substantial correlation with the kinetics of Fas-associated death domain (FADD) and caspase-8 recruitment to lipid rafts. Finally, electron microscopy analysis showed that after CD95 stimulation lipid rafts aggregated in large clusters that were internalized in endosomal vesicles, where caspase-8 underwent massive processing. Taken together, our data demonstrate that CD95 death-inducing signaling complex formation and internalization in type I and type II cells occur in lipid rafts, which are a major site of caspase-8 activation.

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1 Introduction

CD95 (Fas/APO-1) is a major member of the death receptor family, a subgroup of the tumor necrosis factor (TNF) superfamily characterized by a cytoplasmic death domain that is responsible for the transmission of the apoptotic signal [1–3]. The interaction between CD95 and its ligand (CD95L) induces receptor oligomerization, death domain-mediated recruitment of the adaptor protein FADD/MORT1, and binding and activation of procaspase-8 to FADD/MORT1 through their death-effector domains, thereby initiating the caspase cascade responsible for the apoptotic process [4, 5].

Two different CD95-induced apoptosis pathways may be generated in different cell types. Apoptosis in type I cells follows the rapid formation at the death-inducing signaling complex (DISC) of high amounts of active caspase-8, which directly activates the effector caspase-3. By contrast, in type II cells, DISC formation is very slow and insufficient to directly activate caspase-3. In these cells, the apoptogenic activation of mitochondria seems the main pathway responsible for induction of cell death [6, 7].

The ability of CD95L to kill CD95-sensitive cells is exploited in different phases of the immune response including the control of the lifespan of lymphocytes through the induction of apoptosis in activated T and B cells [8, 9]. Lipid rafts are distinct plasma membrane microdomains enriched in cholesterol and sphingolipids [10, 11]. They are more ordered and less fluid than the phospholipid-rich plasma membrane. As a consequence, upon membrane solubilization by non-ionic detergents at low temperatures, rafts remain insoluble and float to low density following gradient centrifugation [12, 13].

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Abbreviations: DISC: Death-inducing signaling complex **GFP:** Green fluorescent protein **MBCD:** Methyl-β-cyclodextrin **Zap-70:** Zeta-associated protein 70

Early signaling events in CD95-induced apoptosis involve caspase-independent formation of receptor microaggregates and caspase-8-dependent formation of larger CD95 aggregates, which are subsequently internalized through an endosomal pathway dependent on actin filaments [14]. CD95 has been suggested to constitutively reside outside lipid rafts in Jurkat T cells and in fibrosarcoma cells [15–17], and both inside and outside this compartment in mouse thymocytes [13]. In several cell types, including primary T cells [18], CD95 stimulation results in the recruitment of Fas-associated death domain (FADD) and caspase-8 to the lipid rafts, suggesting the involvement of lipid rafts in CD95 signaling. In activated T cells, T cell receptor restimulation results in CD95 translocation to lipid rafts and increased sensitivity to CD95-induced apoptosis [19]. However, in human fibrosarcoma cells HT1080, CD95 DISC formation does not occur in lipid rafts, whereas TNFR1 DISC formation occurs in these microdomains and is followed by ubiquitinylation of some DISC proteins, such as TNFR1 itself and receptor-interacting protein (RIP) [17].

The ability of cholesterol-depleting agents to inhibit CD95-induced apoptosis is controversial. Lipid raft disruption by cholesterol depletion has been shown to protect some cell types from CD95-induced apoptosis, supporting again the involvement of these microdomains in death signaling generated by death receptors [16, 18, 20, 21]. Death receptor clustering and recruitment of the DISC components into insoluble plasma membrane fractions suggest that the initial phases of CD95 death signaling occur in lipid rafts. However, following the inability of cholesterol-depleting agents to inhibit CD95 DISC formation and apoptosis in SKW6.4 B lymphoblastoid and H9 T lymphoid cells, the involvement of lipid rafts in CD95 signaling in type I cells has been questioned [14]. We therefore investigated CD95 signaling and DISC formation at the level of subcellular compartments in type I and type II cells.

2 Results

2.1 CD95 expression and apoptosis sensitivity in type I and type II cells

Before investigating the involvement of lipid rafts in CD95 signaling, we analyzed CD95 expression and sensitivity of several human hematopoietic cells. Type II cells are generally less sensitive to CD95 stimulation than type I cells. In order to perform a more reliable comparison between type I and type II signal, the type II cell lines included in this study were selected on the basis of their increased sensitivity to CD95-mediated apoptosis. The B lymphoblastoid JY and the cutaneous T lymphoma HuT78 cells, which behave as type I cells, were compared with human acute T lymphocytic leukemia Jurkat cells and T lymphoblastoid CEM cells, known to be type II cells. All four cell lines were virtually 100% positive for CD95, with JY and HuT78 cells being those with higher receptor density (Fig. 1A). Although at early time points type II cells showed a slightly decreased percentage of apoptosis, CD95 stimulation was able to kill all the four cell lines within 1 day (Fig. 1B), indicating a similar ability to generate apoptotic signaling.

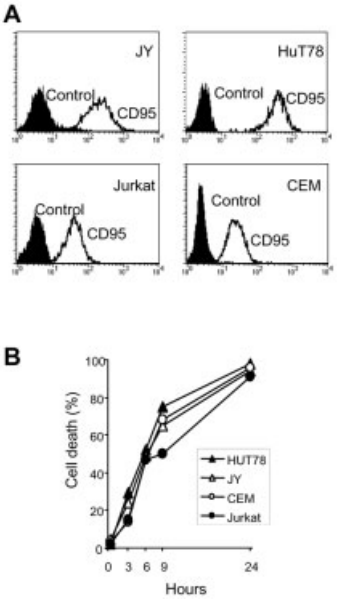

Fig. 1. CD95 expression and apoptosis sensitivity in type I and type II cells. (A) Flow cytometric analysis of type I (JY and HuT78) and type II (Jurkat and CEM) cells stained with phycoerythrin-conjugated control IgG or anti-CD95. (B) Kinetics of CD95-mediated apoptosis in the four cell lines analyzed after treatment with 500 ng/ml anti-CD95 for the indicated time periods. Cell viability was evaluated by ethidium bromide-acridine orange staining and fluorescence microscopy analysis. The data shown are representative of four independent experiments.

2.2 CD95 is constitutively localized in both raft and non-raft cell compartments

After sucrose gradient ultracentrifugation of cell lysates, detergent-resistant lipid rafts float to low-density fractions because of their high lipid content. These insoluble membranes, visible as white opalescent bands in fractions 4 and 5, were compared with soluble fractions 9 and 10 for DISC protein content. The cell lines analyzed did not show significant differences in terms of percentage of proteins present in insoluble fractions, which contained about 1.3% of the total protein content. Immuno-

blot analysis performed on SDS-PAGE loaded with equal amounts of proteins for each sample showed that CD95 was predominantly located in insoluble fractions in JY and HuT78 cells, while it was equally distributed between the compartments in CEM and Jurkat cells (Fig. 2A). As expected, CD4 was detected in Triton X-100-insoluble fractions, whereas the non-raft marker zeta-associated protein 70 (Zap-70) [22] was localized in fractions 9–11 (Fig. 2B).

We next analyzed CD95 distribution by loading equal volumes from each fraction onto the gel, independently of protein concentrations. The raft-associated protein CD4 and the soluble fraction protein Zap-70 were used as controls (Fig. 2C). Although the protein content of the insoluble fractions was up to 50 times lower than that of soluble fractions, the volumetric analysis confirmed that type I cells displayed higher levels of CD95 in lipid rafts (Fig. 2C).

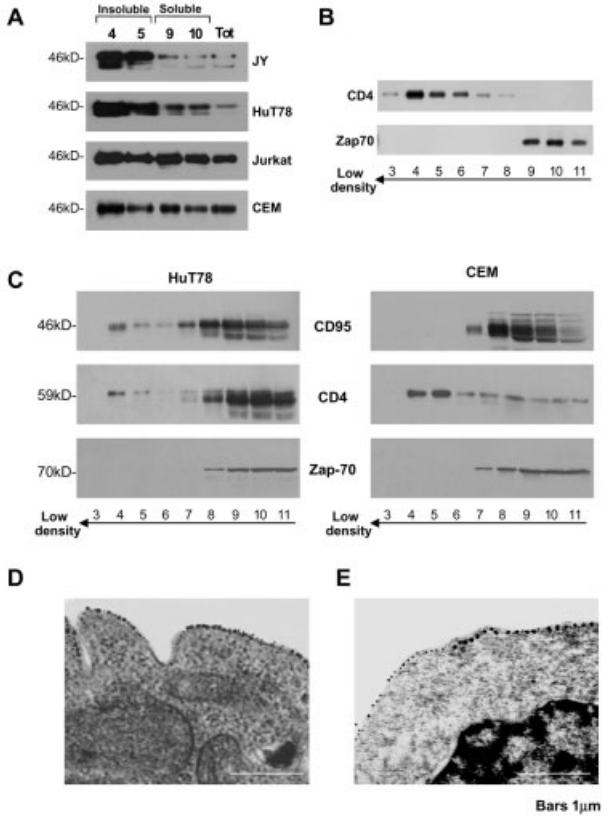

Fig. 2. Different distribution of CD95 in membrane microdomains of type I and type II cells. (A) CD95 immunoblot analysis of Triton X-100-soluble (represented by fractions 9 and 10) and -insoluble (fractions 4 and 5) cell fractions isolated from type I and type II cells. (B) CD4 and Zap-70 immunoblot analysis of Triton X-100-soluble and -insoluble cell fractions obtained from HuT78 cells. Equal amounts of proteins for each fraction were analyzed. (C) CD95, CD4, and Zap-70 immunoblot analysis of Triton X-100-soluble and -insoluble cell fractions obtained from HuT78 and CEM cells. Equal volumes for each fraction were analyzed. (D, E) Electron microscopy analysis of HuT78 (D) and CEM (E) cells labeled with anti-CD95 and anti-CD4 antibodies and revealed by 4-nm (CD95) and 25-nm (CD4) immunogold particles. A representative experiment of four is shown.

To confirm the immunoblot data concerning the different plasma membrane sublocation of CD95 in type I and type II cells, we performed immunogold double staining to localize CD95 and the raft-resident CD4 antigen [23] simultaneously in native HuT78 and CEM cells. CD95 and CD4 co-localized in the plasma membrane of unstimulated HuT78 cells (Fig. 2D), while in CEM cells CD95 displayed a diffuse localization throughout the cell surface and did not extensively co-localize with CD4, which exhibited a focal localization (Fig. 2E).

2.3 Recruitment of the DISC to lipid rafts in type I and type II cells

We then analyzed whether the distribution of CD95 was affected by receptor stimulation. HuT78 and CEM cells were stimulated with agonistic anti-CD95 antibody, cell lysates fractionated, and CD95 distribution evaluated by immunoblot analysis. We found that CD95 was recruited to the insoluble fractions after receptor stimulation with similar kinetics in the two cell types, while the pattern of the control protein CD4 was not affected by CD95 triggering (Fig. 3A).

To better determine the extent of CD95 recruitment to lipid rafts in type I and type II cells, in comparison with other raft proteins, we performed densitometric quantification of insoluble fraction immunoblot bands for CD95 and CD4 (Fig. 3B). The same cell fractions were then analyzed by Western blotting for the presence of DISC components. FADD/MORT1 and caspase-8 were found predominantly in the soluble fractions of unstimulated cells, but were rapidly recruited to the insoluble fractions in type I cells after CD95 cross-linking (Fig. 3C). Interestingly, recruitment of both FADD/MORT1 and caspase-8 to the insoluble fractions in CEM cells was slower, requiring several hours of stimulation, as indicated by the different ratios of pro-caspase-8 (p55–53) to processed caspase-8 (p41–43) (Fig. 3C). Similar results were obtained in type I and type II cells stimulated with recombinant CD95 ligand (data not shown).

To further demonstrate that the early events of CD95 signaling occur in lipid rafts, we immunoprecipitated CD95 of soluble and insoluble fractions from the same samples used for Western blotting analysis shown in Fig. 3A, C.

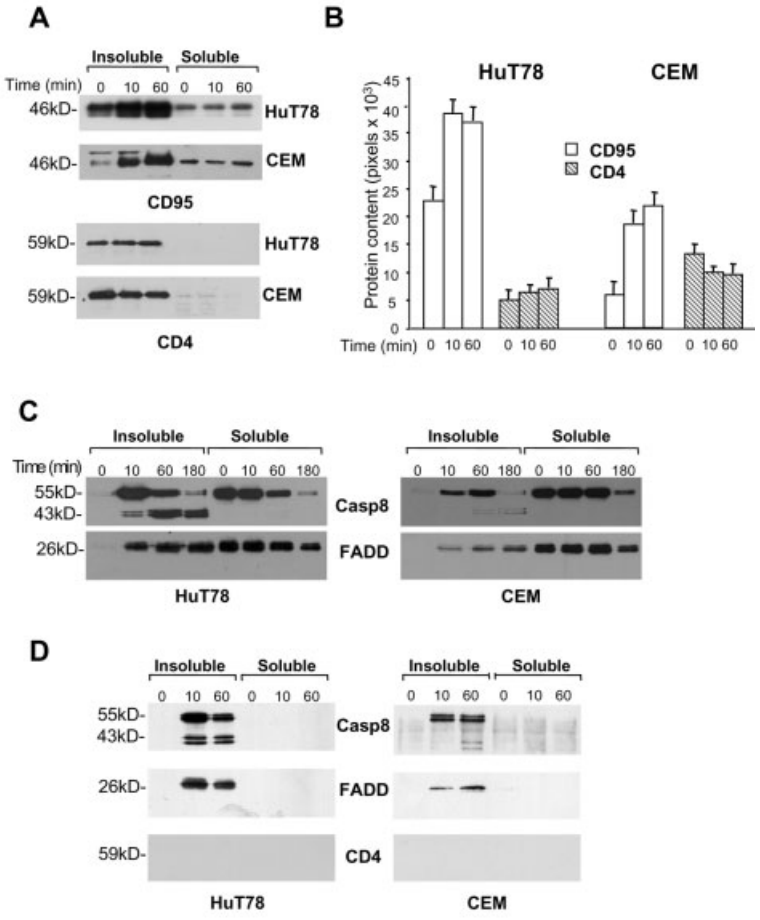

Fig. 3. Recruitment of DISC components to lipid rafts upon CD95 stimulation in type I and type II cells. (A) Immunoblot analysis of CD95 and CD4 in insoluble and soluble fractions of HuT78 and CEM cells stimulated with anti-CD95 for the indicated time points. (B) Densitometric quantification of CD95 and CD4 immunoblot bands in insoluble fractions of cells treated as in (A). Bands have been quantified with NIH-Image program and protein content is indicated as arbitrary units (pixels $\times 10^3$). Data represent means ± SD of three independent experiments. (C) Immunoblot analysis of caspase-8 and FADD/MORT1 in soluble and insoluble fractions of HuT78 and CEM cells. (D) Immunoprecipitation of CD95 DISC from insoluble and soluble fractions of unstimulated or CD95-stimulated Hut78 and CEM cells. CD95 was immunoprecipitated and blots probed for FADD, caspase-8 (left and center), and CD4 (top right). Lysates of each cell fraction before DISC immunoprecipitation were probed for CD4, both from HuT78 (bottom right) and CEM (data not shown) cells. A representative experiment of four is shown. Cells were stimulated with anti-CD95 for the indicated times.

FADD, pro-caspase-8 and processed caspase-8 co-immunoprecipitated with CD95 only from cell fractions corresponding to lipid rafts of stimulated cells, but not from their soluble fractions. DISC formation was rapid and extensive in type I cells, and delayed and moderate in type II cells (Fig. 3D). As expected, the raft-associated protein CD4 did not co-immunoprecipitate with CD95 (Fig. 3D, bottom), indicating that the immunoprecipitation was specific for DISC components. Therefore, the different extent of caspase-8 recruitment and activation in lipid rafts of type I and type II cells correlated with the respective ability to form the DISC, suggesting that lipid rafts are the first site of caspase-8 activation after CD95 stimulation.

2.4 Recruitment of CD95 DISC to lipid rafts is not dependent on caspase activation

To evaluate whether CD95 recruitment to lipid rafts was dependent on caspase activation, we analyzed CD95 and caspase-8 content of soluble and insoluble fractions isolated from type I and type II cells pretreated with the pan-caspase inhibitor zVAD before CD95 stimulation. We found that zVAD pretreatment did not prevent CD95 and caspase-8 translocation to lipid rafts following stimulation, indicating that the recruitment of DISC components to lipid rafts is not caspase dependent. As expected, the pan-caspase inhibitor zVAD prevented the processing of caspase-8 (Fig. 4A).

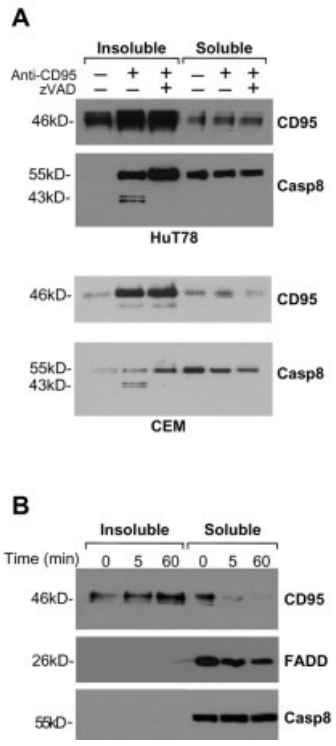

Fig. 4. Caspase activation is not required for DISC assembly into lipid rafts of type I and type II cells. (A) Immunoblot analysis of CD95 and caspase-8 in soluble and insoluble fractions of HuT78 and CEM cells treated with anti-CD95 and the caspase inhibitor zVAD (40 μM) where indicated. HuT78 and CEM cells were stimulated for 30 and 180 min, respectively. (B) Immunoblot analysis of insoluble and soluble fractions obtained from a CD95-stimulated clone of HuT78 cells expressing both wild-type and death domain-defective CD95 receptors. A representative experiment of three is shown.

To determine whether FADD/MORT1 and caspase-8 recruitment to lipid rafts was dependent on their ability to bind cross-linked oligomeric CD95, we analyzed the HuT78 CD95-resistant clone B12. These cells express both wild-type receptor and a mutated CD95 lacking the death domain, and are unable to form the DISC [24]. Despite the inability to generate a death signal, full-length CD95 was recruited to lipid rafts after stimulation of B12 cells (Fig. 4B), suggesting that DISC formation is not required for recruitment of CD95 to lipid rafts. In contrast, an intact CD95 death domain was necessary for FADD/MORT1 and caspase-8 recruitment to lipid rafts, since their translocation was abolished in the presence of mutant receptors (Fig. 4B). Thus, the inability to recruit FADD/MORT1 and caspase-8 to lipid rafts correlates with resistance to CD95-induced apoptosis, confirming the relevance of DISC formation in lipid rafts for the apoptotic signal.

2.5 Treatment with cholesterol-depleting agents does not modify the association of CD95 with lipid rafts in type I cells

Cholesterol-depleting agents are reportedly unable to inhibit CD95 clustering, internalization, and CD95-induced apoptosis in type I cells [14]. Accordingly, we did not observe decreased apoptosis sensitivity in cells treated with cholesterol-binding compounds (data not shown). Therefore, we investigated the effect of the cholesterol-depleting agent methyl-β-cyclodextrin (MBCD) on CD95 association with lipid rafts in HuT78 cells. In agreement with literature data [25], staining of plasma membrane lipids with PKH26 linker showed that Triton X-100-resistant membranes were affected by MBCD treatment, which resulted in the formation of small cholesterol-deprived lipid rafts (Fig. 5A). Western blot analysis showed that some lipid raft-associated proteins located in cholesterol-rich regions, such as Thy1 and Fyn, were released from insoluble fractions of MBCD-treated cells (Fig. 5B and data not shown), con-

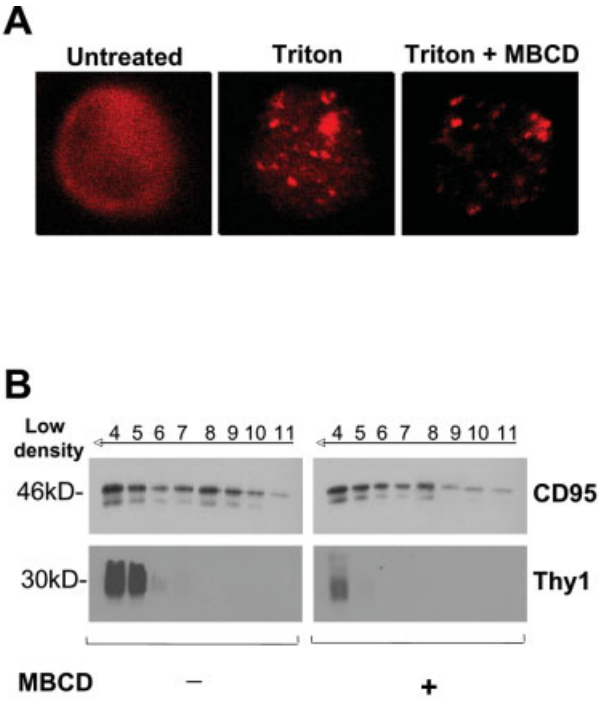

Fig. 5. Cholesterol depletion does not modify CD95 association with lipid rafts in HuT78 cells. (A) Immunofluorescence analysis of untreated, Triton X-100-treated, or Triton X-100- and MBCD-treated HuT78 cells, stained with the lipid-binding Rhodamine-conjugated PKH26 linker. (B) Immunoblot analysis showing CD95 and Thy1 distribution in insoluble and soluble cell fractions isolated from control and MBCD-treated HuT78 cells. The same blot has been used for detecting the two proteins. A representative experiment of three is shown.

firming the effective removal of cholesterol from the plasma membrane. In contrast, the relative amount of CD95 receptor localized in lipid rafts was not significantly affected by MBCD treatment of type I cells (Fig. 5B). These results suggest that a considerable amount of CD95 molecules might be localized in the MBCD-resistant sphingolipid-rich core of lipid rafts, thus providing an explanation for the inefficacy of MBCD to protect some cell types from CD95-induced cell death.

2.6 Type I-"like" or type II-"like" CD95 signaling depends on CD95 levels in HepG2 cells

In order to determine whether the differences in CD95 DISC assembly into the lipid rafts observed in the type I and type II cells were dependent on the different levels of CD95 present on the cell surface, we transduced the type II hepatoma HepG2 cells [26] with a retroviral vector carrying the CD95 cDNA (HepG2/CD95). The levels of surface CD95 expressed on HepG2 and HepG2/CD95 cells were similar to the levels of CD95 observed for CEM and Hut78 cells, respectively (Fig. 6A). We then isolated lipid rafts from the two HepG2 cell populations and analyzed CD95 distribution in soluble and insoluble cell fractions by Western blotting. c-Src and transferrin receptor were used as raft- and non-raft-associated proteins, respectively. In HepG2 cells, CD95 was present both in soluble and insoluble cell fractions, while the HepG2/CD95 cells displayed an increased localization of CD95 in insoluble fractions, resembling type I cells (Fig. 6B). Accordingly, CD95 triggering resulted in a rapid caspase-8 recruitment and activation in HepG2/CD95 cells, as compared with control HepG2 cells (Fig. 6C), suggesting that the amount of CD95 available for DISC formation is critical for type I signaling.

Anti-apoptotic Bcl-2 family members, such as Bcl-2 or Bcl-xL, are able to protect type II cells from CD95-induced apoptosis. In order to confirm that high CD95 expression can transform type II into type I cells, we overexpressed Bcl-xL in HepG2 and in HepG2/CD95 cells and evaluated its ability to protect from CD95-induced apoptosis. As expected for type II cells, Bcl-xL overexpression protected HepG2 cells from CD95-mediated apoptosis. In contrast, high Bcl-xL expression did not protect HepG2/CD95 cells from CD95 stimulation (Fig. 6D), indicating that they behaved as type I cells. Thus, the levels of CD95 expression are critical for the type of signaling generated after receptor stimulation.

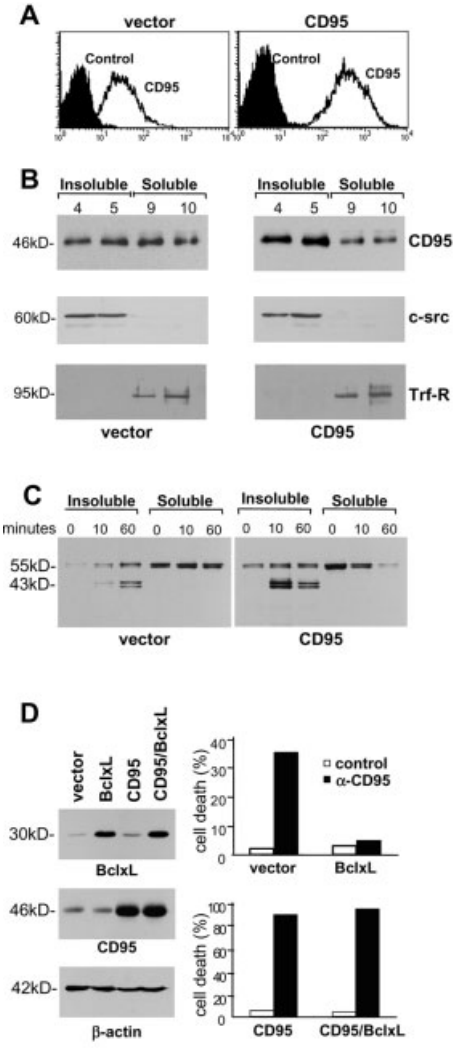

Fig. 6. Type I-"like" or type II-"like" CD95 signaling depends on CD95 levels in HepG2 cells. (A) Flow cytometric analysis of CD95 in HepG2 cells transduced with empty GFP$^-$PINCO (Vector) or with GFP$^-$PINCO/CD95 (CD95). Cells were stained with phycoerythrin-conjugated control IgG or anti-CD95 as indicated. (B, C) Insoluble and soluble fractions of HepG2 cells transduced as in (A) were analyzed by immunoblotting for CD95, the raft protein c-Src and the soluble fraction-associated transferrin receptor (B), and caspase-8 before and after 10 and 180 min of CD95 stimulation (C). (D) Western blotting for Bcl-xL, CD95, and β-actin in HepG2 cells transduced as indicated (left). Quantitative analysis of CD95-induced apoptosis of the different HepG2 cell populations transduced as indicated (right).

2.7 Recruitment of the DISC to lipid rafts of primary T cells

Activated T lymphocytes have been described as type I cells. We evaluated CD95 signaling in short-term (day 1)- and long-term (day 6)-activated T lymphocytes. The

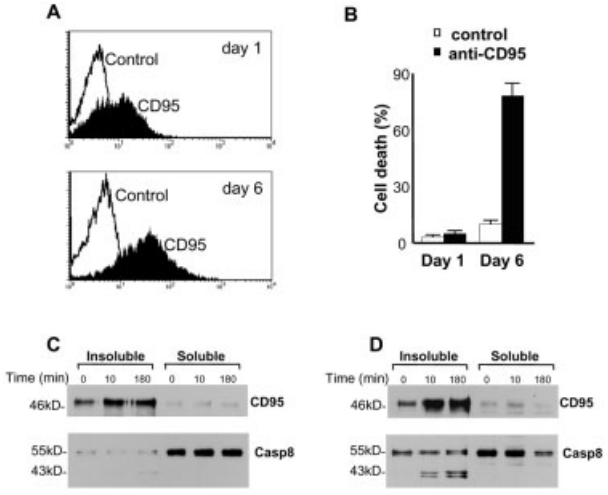

Fig. 7. CD95 DISC formation in lipid rafts of activated T cells. (A) Flow cytometric analysis of CD95 expression in day-1- and day-6-activated T lymphocytes. (B) Percentage of cell death in day-1- and day-6-activated T lymphocytes stimulated for 24 h with anti-CD95. (C, D) Immunoblot analysis of CD95 and caspase-8 in soluble and insoluble fractions of untreated and anti-CD95 stimulated day-1 (C) and day-6 (D) T lymphocytes. Cells were stimulated with anti-CD95 for the indicated times.

expression of CD95 was low in resting T cells, but progressively increased following PHA and IL-2 stimulation (Fig. 7A and results not shown). In agreement with literature data, CD95 stimulation resulted in apoptosis induction only in long-term-activated T cells, while day-1 cells were almost insensitive to CD95-induced apoptosis (Fig. 7B). Although CD95 was recruited to lipid rafts after stimulation with a rapid kinetics in both day-1 and day-6 T cells, caspase-8 was recruited to lipid rafts very weakly and slowly in day-1 T cells (Fig. 7C), while long-term-activated T cells displayed a rapid and massive caspase-8 recruitment and processing (Fig. 7D). Thus, a rapid recruitment and processing of caspase-8 in lipid rafts is associated with the acquired sensitivity to CD95-mediated apoptosis of activated T cells.

2.8 CD95 internalization is delayed in type II cells

CD95 stimulation in type I cells is followed by rapid receptor internalization [14]. Little is known about CD95 internalization in type II cells. Since these cells displayed a weak and particularly delayed DISC assembly into lipid rafts, we investigated the subcellular distribution of CD95 at early and late time points in the two cell types by immunofluorescence studies. In unstimulated cells, CD95 was uniformly distributed on the plasma membrane of both HuT78 and CEM cells, although the former exhibited higher expression of the receptor. Ten minutes after stimulation, CD95 showed a polarized distribution in the two cell lines, suggesting that the formation of CD95 macroaggregates occurs rapidly in both cell types. However, after 30 min, CD95 was mainly cytoplasmic in most of the HuT78 cells, while it maintained the polarized surface distribution in CEM cells (Fig. 8A). Thus, while we could detect CD95 capping and internalization in type I cells, type II cells displayed CD95 capping but not internalization at early time points. In contrast, we found extensive cytoplasmic localization only after 3 h of CD95 cross-linking in CEM cells (Fig. 8B–D), indicating that receptor internalization is a late event in type II cells.

The cytoplasmic localization of CD95 in stimulated cells was confirmed by exposing stimulated cells to pH 2.5, which removes the receptor-antibody complexes located on the cell surface. While all the surface fluorescence was lost in unstimulated cells exposed to low pH, stimulated cells maintained a clear intracellular labeling, confirming that a substantial amount of CD95 was internalized (Fig. 8E). Similar results were obtained with recombinant CD95 ligand stimulation (Fig. 8F). The different kinetics of CD95 internalization observed in type I and type II cells correlated with the time of FADD and caspase-8 recruitment to lipid rafts, suggesting that DISC formation in lipid rafts may be related to the CD95 internalization process.

2.9 CD95 triggering induces the formation and internalization of receptor-containing raft macroaggregates

In order to explore the potential role of lipid rafts in CD95 clustering and internalization, we used electron microscopy to localize CD95 and the canonical raft-associated protein CD4, before and after stimulation. In unstimulated HuT78 cells, CD95 was distributed throughout the plasma membrane (Fig. 9A). However, receptor cross-linking resulted in immediate polarization of CD95, which co-localized in the plasma membrane with CD4 (Fig. 9B, D) as a likely result of the coalescence of multiple CD95-containing lipid rafts. Moreover, shortly after CD95 stimulation, CD95 became almost completely internalized in the majority of these cells and localized in structures resembling endosomal vesicles (Fig. 9C, E). CD4 was present within the same vesicles, indicating that CD95 was internalized in raft aggregates. Aliquots of these samples were analyzed also by Western blotting for caspase-8 activation at early time points (*i.e.* before CD95 internalization). We found that in comparison with longer stimulation times (see Fig. 3), caspase-8 processing within 30 min from CD95 stimulation was limited (Fig. 9F). Since electron and confocal microscopy

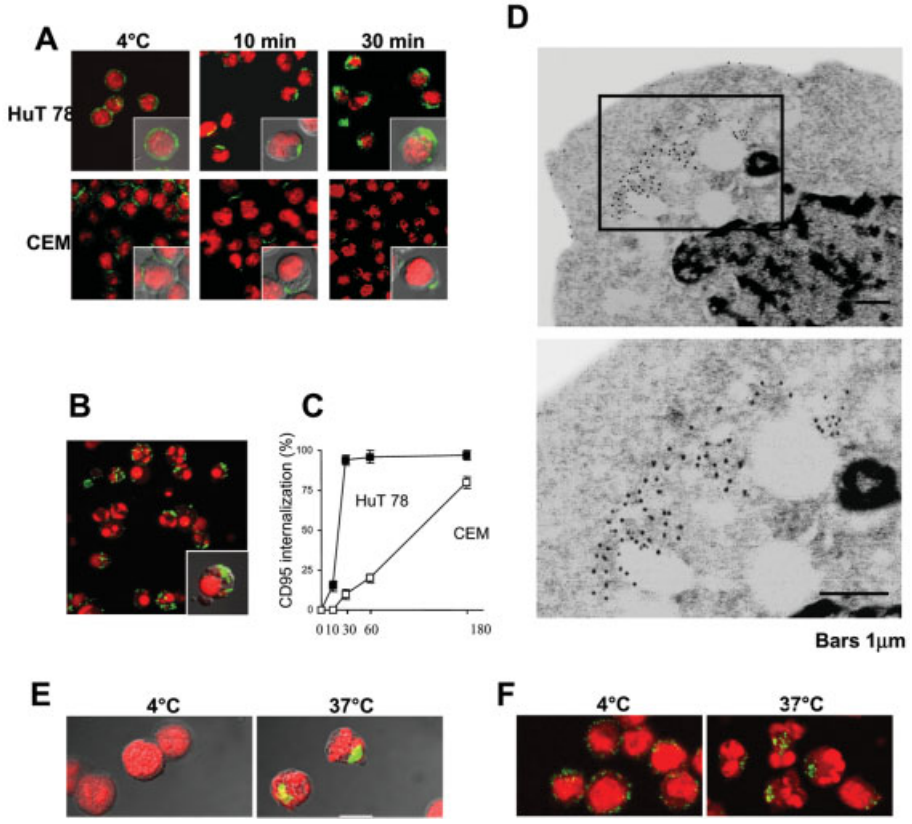

Fig. 8. CD95 internalization in HuT78 and CEM cells. Immunofluorescence analysis showing CD95 localization in untreated, 10-min-, and 30-min-stimulated HuT78 and CEM cells (A) or 180-min-stimulated CEM cells (B). Nuclei were stained with 0.1 μg/ml propidium iodide after cell permeabilization. Samples were analyzed by confocal microscopy. The data shown are representative of three independent experiments. (C) Kinetics of CD95 internalization in HuT78 and CEM cells. The percentage of cells displaying internalized CD95 is shown at the indicated time points. Data are means + SD of three independent experiments. (D) Electron microscopy analysis of CEM cells stimulated for 180 min with anti-CD95 agonistic antibody. CD95 labeling was performed with 4-nm gold particle-conjugated anti-IgM antibody. (E, F) Immunofluorescence analysis of internalized CD95 in HuT78 cells treated at 4°C or 37°C with anti-CD95 and anti-IgM FITC and then exposed to pH 2.5 before fixation (E) or with recombinant Flag-tagged CD95L, anti-Flag antibody, and FITC goat anti-mouse antibody (F). Data are representative of three independent experiments.

showed that virtually all HuT78 cells internalized the DISC within 30 min after CD95 triggering, it is likely that internalized lipid rafts are the major site of caspase-8 activation in type I cells.

3 Discussion

In this study we demonstrate that early CD95 signaling in type I and type II cells occurs primarily in lipid rafts, which consistently appeared to be the major site of DISC formation and caspase-8 activation. CD95 is preferentially located in lipid rafts in type I cells. Although the physical requirements for CD95 partitioning into lipid rafts is unknown, it does not appear to depend on its cytoplasmic domain, as mutants containing as little as six amino acid residues below the endoplasmic leaflet of the membrane did not differ from the wild-type receptor in terms of membrane distribution (results not shown). The different localization pattern may theoretically influence the death pathway generated by ligand binding. However, type I and type II cells do not differ in terms of CD95 localization after receptor stimulation, suggesting that other factors are responsible for the different extent of DISC formation observed in type I and type II cells.

The ability of type I cells to recruit FADD/MORT1 and caspase-8 to the DISC immediately after CD95 stimulation parallels the recruitment of these proteins to lipid rafts. Type II cells are unable to form a significant CD95 DISC after stimulation [6, 27]. Accordingly, we found that shortly after CD95 stimulation FADD/MORT1 and caspase-8 are weakly recruited to lipid rafts in CEM and Jurkat cells, further confirming that such recruitment is a

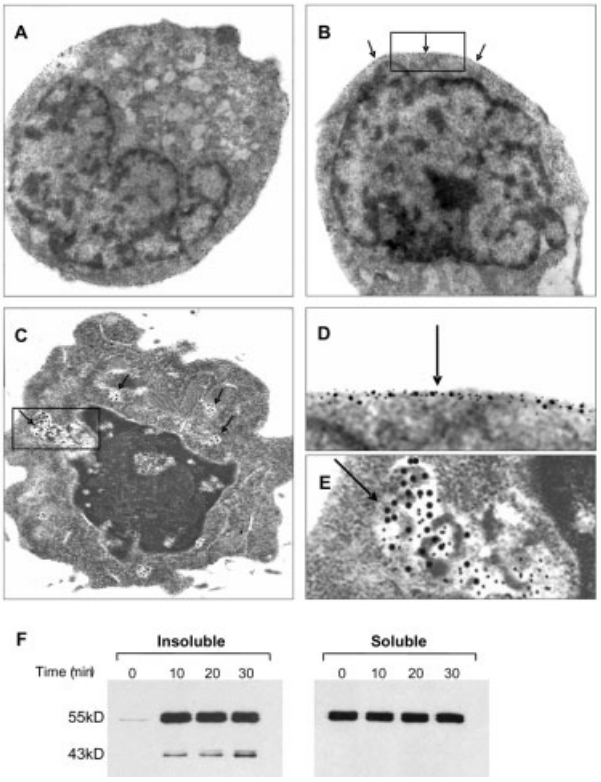

Fig. 9. Formation and internalization of CD95-containing raft macroaggregates following CD95 triggering in type I cells. Electron microscopy analysis showing CD4 and CD95 localization in HuT78 cells, untreated (A) or stimulated with anti-CD95 for 10 (B) or 20 min (C). Double labeling was performed with 4-nm immunogold particles for CD95 and 15-nm (A, B) or 25-nm (C) immunogold particles for CD4. Arrows indicate polarization (B) and endocytosis in endosomal vesicles (C) of CD4 and CD95 after receptor stimulation. (D) Higher magnification of the area highlighted in (B). (E) Higher magnification of the area highlighted in (C). (F) Pattern of caspase-8 activation in surface and internalized lipid rafts after CD95 stimulation. Immunoblot analysis of insoluble and soluble fractions from HuT78 cells, showing the kinetics of caspase-8 recruitment and processing after CD95 triggering. Single samples were split and simultaneously analyzed by both electron microscopy and immunoblotting. A representative experiment of four is shown.

specific event and that DISC formation occurs in lipid rafts. Interestingly, however, we found high levels of FADD/MORT1 and procaspase-8 after 3 h of stimulation in lipid rafts of type II cells. Thus, it is possible that substantial DISC formation occurs in some type II cells, but with considerably delayed kinetics and slow caspase-8 processing.

After stimulation, CD95 is rapidly internalized together with lipid rafts in type I cells. This internalization requires caspase-8 activation and actin filaments [14]. Although its functional significance is not clear, we did not observe a significant decline in CD95 levels in the 6 h following stimulation (results not shown), suggesting that CD95 internalization does not promote its degradation. Moreover, kinetics analysis indicates that a substantial activation of caspase-8 occurs after DISC internalization. Thus, the presence of active caspase-8 in selected intracellular compartments may favor the proteolytic cleavage of relevant substrates during type I apoptotic signaling. As observed for FADD and caspase-8 recruitment to lipid rafts, CD95 internalization was a late event in CD95 stimulated type II cells, thus suggesting that CD95 internalization might be related to DISC assembly at lipid rafts also in type II cells. The strict correlation between the intensity of CD95 expression and the kinetics of DISC formation and internalization suggests that the level of receptor density in some circumstances may determine the quality of the death signal.

The role of lipid rafts in CD95 signaling has been recently questioned following the failure of cholesterol-depleting agents to inhibit receptor internalization and apoptosis in SKW6.4 B lymphoblastoid and T lymphoma H9 type I cells, raising the possibility that disruption of insoluble membrane microdomains may affect CD95-induced apoptosis only in type II cells [14]. However, although cholesterol-depleting agents reduce the size of lipid rafts, some receptors are not displaced from this compartment after cholesterol depletion [25, 28, 29]. Indeed, we found that CD95 association with lipid rafts was moderately influenced by cholesterol-depleting agents in type I cells, while other receptors were rapidly released from this compartment.

In conclusion, our results indicate that lipid rafts and their macroaggregates may represent key sites for the propagation of early signaling events generated by CD95 stimulation in lymphoid cells. It is likely that a more focused study of this compartment will provide valuable information on the mechanisms regulating CD95 function in a variety of physiological and pathological systems.

4 Materials and methods

4.1 Cell cultures and flow cytometry analysis

The human T lymphoblastoid cell line CCRF-CEM, the human cutaneous T lymphoma cell line HuT78, the human acute T cell leukemia cell line Jurkat, and the human B lymphoblastoid cell line JY were grown in RPMI (Life Technologies Inc., Grand Island, NY) supplemented with 10% FBS (Life Technologies Inc.), 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine (Gibco BRL, Rockville, MD)

and maintained in 5% CO_2 at 37°C. The human hepatoma HepG2 cells were grown in Dulbecco's modified essential medium (Life Technologies Inc.) supplemented as described above. To detect surface CD95 expression, cells were stained with control or anti-CD95 antibodies (DX2; DAKO, Glostrup, Denmark) and analyzed by FACScan (Becton Dickinson, San Jose, CA). Peripheral blood T lymphocytes were cultured for 2 days in the presence of 1 μg/ml PHA and 600 U/ml IL-2. The day-3 cells were washed and cultured in the presence of 600 U/ml IL-2 until day 6.

4.2 CD95 stimulation and isolation of lipid rafts

One hundred million cells were left untreated or stimulated for the indicated time points with 1 μg/ml CH11 antibody (Upstate Biotechnology, Lake Placid, NY). Pre-incubation (30 min) with 40 μM zVAD-fmk (Bachem, Bubendorf, Switzerland) was performed where indicated. An aliquot of treated samples was routinely collected to subsequently evaluate cell viability as described [30]. Lipid raft extraction was performed according to standard protocols [23, 31]. Briefly, cell pellet was dissolved in 750 μl of 1% Triton X-100/ 25 mM MES/150 mM NaCl buffer at 4°C. After homogenization, cell lysates were subjected to sucrose gradient centrifugation at 45,000 rpm for 16 h in an SW60 rotor (Beckman Instruments). Twelve 375-μl fractions were collected from the top to the bottom of each gradient. For cholesterol depletion studies, cells were treated with 10 mM MBCD for 15 min at 37°C in serum-free medium, before lipid raft isolation.

4.3 Western blotting and immunoprecipitation

Equal amounts of proteins or equal volumes for each sample were used for immunoblot studies. Rabbit polyclonal anti-CD95 C20 and anti-transferrin receptor (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-FADD/ MORT1 (Becton Dickinson Transduction, Los Angeles, CA), mouse monoclonal anti-caspase-8 (clone 5F7), anti-Zap-70 and anti c-Src (Upstate Biotechnology), anti-Thy1 (PharMingen, San Diego, CA), and anti-CD4 (Novocastra, Newcastle, GB) antibodies were used. Immunoprecipitation studies were performed as previously described [32].

4.4 Isolation, cloning, and sequencing of CD95-resistant clones

HuT78 cells were chronically stimulated with CH11 antibody. Apoptosis-resistant cells were cloned by limiting dilution and grown in the absence of anti-CD95 antibody. mRNA was extracted (Dynabeads mRNA Direct Kit; Dynal, Oslo, Norway) and reverse-transcribed with oligo(dT) primer and Superscript II reverse transcriptase (Gibco). The CD95-coding region was cloned and six different positive clones were subjected to automated sequencing.

4.5 Transduction of HepG2 cells

Flag-tagged CD95 cDNA was cloned into a green fluorescent protein (GFP)-defective version of the PINCO retroviral vector and Bcl-xL cDNA was cloned into the GFP-containing PINCO vector. Cells were infected as previously described [33]. Gene transfer efficiency was evaluated by flow cytometry analysis, based on the expression of the GFP reporter protein and on the anti-Flag positivity (anti-flag M2 antibody; Sigma, St. Louis, MO).

4.6 Electron microscopy

Three million cells, either untreated or stimulated with 1 μg/ml CH11 antibody were fixed with 2% paraformaldehyde/ 1% glutaraldehyde and embedded in Epon 812. Samples were then thin-sectioned and double-stained for CD95 and CD4. CD4 was detected by biotinylated anti-CD4 antibody (Caltag, Burlingame, CA) and 15- or 25-nm-diameter gold particle-conjugated streptavidin (Amersham Pharmacia Biotech, Piscataway, NJ). For CD95, 4-nm-diameter gold particle-conjugated anti-mouse IgM antibodies were used.

4.7 Internalization assay and immunofluorescence

One million HuT78 and CEM cells were incubated with 5 μg/ml of CH11 antibody for 45 min on ice. Cells were than stained with FITC-conjugated anti-mouse antibody for 30 min on ice. After washing, cells were kept at 37°C for the indicated time points to trigger CD95 stimulation. Cells were fixed with 2% paraformaldehyde for 20 min at 37°C and permeabilized with 0.1% Triton X-100/ PBS for 3 min at room temperature. Nuclei were stained with 0.1 μg/ml propidium iodide. Samples were transferred to poly-D-lysine-coated glass coverslips and visualized with an Olympus confocal microscope. For CD95 ligand-induced CD95 internalization, HuT78 cells were labeled using recombinant Flag-tagged Super FasL (Alexis, Germany), mouse anti-Flag antibody, and fluorescein-linked goat anti-mouse antibody (Molecular Probes, Eugene, OR).

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Correspondence: Ruggero De Maria, Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, viale Regina Elena 299, I-00161 Rome, Italy
Fax: +39-0649387087
e-mail: rdemaria@tin.it

