

Chapter 2

CD95 Stimulation with CD95L and DISC Analysis

Matthieu Le Gallo and Patrick Legembre

Abstract

CD95 and its ligand CD95L play a major role in immune surveillance and homeostasis. CD95L is expressed by activated T lymphocytes and NK cells to induce apoptosis in cancer and virus-infected cells. The goal of this chapter is to describe a method used to immunoprecipitate CD95 and analyze its associated protein complex in cells stimulated with a cytotoxic CD95L (i.e., Ig-CD95L).

Key words Fas, FasL, Death receptors, Apoptosis, Immunoprecipitation

1 Introduction

Apoptosis is an essential mechanism in tissue homeostasis and elimination of infected cells and tumor cells by the immune system. This cellular mechanism can be initiated by the activation of death receptors expressed at the surface of target cells. All death receptors belong to the *tumor necrosis factor* (TNF) receptors superfamily. CD95, also known as Fas, is a member of this superfamily and this receptor is ubiquitously expressed. By contrast, its ligand, CD95L (also known as FasL) is mainly expressed at the surface of the activated T lymphocytes and NK cells. CD95 is a 40–50 kDa transmembrane receptor that mediates apoptosis when interacting with its ligand. Upon binding of CD95L to CD95, the death receptor assembles the *Death inducing signaling complex* (DISC). DISC was first described by Peter's group in 1995 [1]. This complex consists of the adaptor protein FADD, which recruits the pro-apoptotic proteases caspase 8 and caspase 10 and a pseudo-caspase, namely cFLIP. An increasing number of signaling proteins has been shown to bind the DISC and modulate its formation and activity [1]. The DISC molecules interact through homotypic contacts, though the complete stoichiometry of the DISC is not fully understood. The DISC is a central regulator of the CD95 signaling pathway inducing both conventional apoptotic pathways and non-apoptotic pathway like PI3K, ERK, and NF- κ B [2–9].

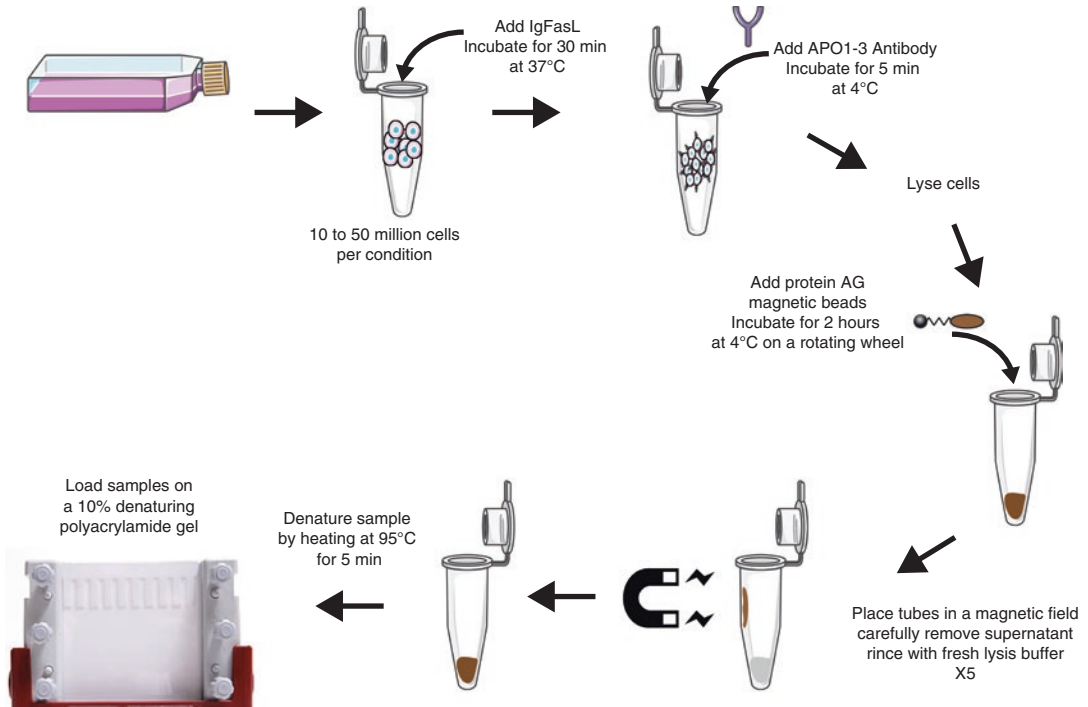


Fig. 1 Schematic description of the DISC immunoprecipitation steps

Several methods have been already published to analyze the CD95-DISC *in vitro* but most of them, describe a complex formed in presence of the agonistic monoclonal antibody (mAb) APO1.3 [10]. In this protocol, we describe methods used in the laboratory to study DISC formation in cells stimulated with either agonistic anti-CD95 mAb or with Ig-CD95L, a recombinant CD95L that mimics the cytotoxic membrane-bound CD95L. Ig-CD95L corresponds to a fusion between the Ig-like domain of the human leukemia inhibitory factor (LIF) receptor gp190, a linker and the extracellular domain of CD95L (*see* Chapter 1 and [11]).

Herein, we describe our method to immunoprecipitate DISC (Fig. 1) and characterize its composition. First, target cells are stimulated with either Ig-CD95L or agonistic anti-CD95 antibody APO1-3 to induce DISC formation. Then, cells are lysed and finally immunoprecipitation is performed.

2 Materials

All solutions must be prepared with ultrapure water and analytical grade reagents. All buffers must be sterilized using 0.22 μ m filters.

Solutions can be stored at +4 °C and are considered stable for a week.

2.1 Equipment

1. Cell culture flasks.
2. Microcentrifuge capable of $21,500 \times g$.
3. Rotating wheel.
4. Western blotting equipment.
5. 1.5 mL eppendorf tubes.
6. Nitrocellulose membranes.
7. Magnetic IP tube holder.

2.2 Reagents

1. RPMI media supplemented with 8 % fetal calf serum (FCS).
2. Lysis buffer to be prepared on ice before starting the experiment: 1 % Triton X-100, Protease inhibitor cocktail (Sigma, France) and Phosphatase inhibitor cocktail (Sigma) have to be added to the 1× Hepes buffer before use.
3. 1× Hepes buffer: 25 mM Hepes, 150 mM NaCl, 1 mM NaF, 1 mM NaVO_4 , 2 mM EGTA; pH must be adjusted to 7.4; filtered solution can be stored at $+4^\circ\text{C}$ for a month.
4. 1× phosphate buffer saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 .
5. 1× Tris buffered saline–Tween (TBS–T): 160 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.05 % Tween 20.
6. Resolving gel buffer: 1.5 M Tris base, pH 8.8; weigh 91 g of Tris base (121.14 g/mol) and transfer to a glass beaker; add ultrapure water to a volume of 400 mL; mix and adjust pH with HCl (37%). Complete the volume to 500 mL with water and store at 4°C .
7. Stacking gel buffer: 0.5 M Tris base, pH 6.8; weigh 15 g of Tris base (121.14 g/mol) and prepare a 250 mL solution as aforementioned. Store at 4°C .
8. Acrylamide 40 % mix 29:1.
9. TEMED (*N,N,N,N'*-tetramethyl-ethylenediamine).
10. Ammonium persulfate (APS): 10 % solution in water, store at -20°C .
11. Protein AG-magnetic beads (Ademtech, Pessac, France).

2.3 Antibodies

2.3.1 Primary Antibodies

1. CD95 mouse monoclonal antibody [clone APO1-3] (Epigentek, Mundolsheim, France).
2. CD95 (C-20) rabbit polyclonal antibody (Santa Cruz, Heidelberg, Germany).
3. c-FLIP mouse IgG1 monoclonal antibody [clone 7F10] (Enzo Life Sciences, Villeurbanne, France).
4. Caspase-8 mouse IgG2b monoclonal antibody [clone 5F7] (Enzo Life Sciences, Villeurbanne, France).
5. FADD mouse IgG1 monoclonal antibody [clone A66-2] (BD Pharmingen, Allschwil, Switzerland).

2.3.2 Secondary Antibodies

1. Goat anti-mouse IgG1 HRP conjugated (SouthernBiotech, Nanterre, France).
2. Goat anti-mouse IgG2b HRP conjugated (SouthernBiotech, Nanterre, France).
3. Goat anti-rabbit polyclonal antibody HRP conjugated (Dako, Les Ulis, France).
4. Ig-CD95L solution (ultracentrifuged and filtered media from Ig-CD95L-transfected HEK 293T cells) (cf Chapter 1).

3 Methods

All steps must be performed on ice unless otherwise noted.

Before starting the experiment, the centrifuge has to be cooled down to +4 °C.

Label the tubes, one per condition. Precool the tubes by placing them on ice (*see* **Note 1**) for at least 30 min.

3.1 Stimulation

1. Harvest 10^7 cells (Type I cells) or 5×10^7 cells (Type II) per condition (*see* **Note 2**).
2. Wash cells with fresh media.
3. Centrifuge cells at $400 \times g$ for 5 min, discard supernatant.
4. Resuspend cells in 1 mL of culture medium per condition.
5. Add Ig-CD95L (100 ng/mL) and incubate at +37 °C for different time points.

3.2 Labeling

1. Centrifuge cells at $400 \times g$ for 5 min at +4 °C.
2. Discard supernatant.
3. Wash pellet with 1 mL ice-cold PBS.
4. Centrifuge at $400 \times g$ for 5 min at +4 °C.
5. Resuspend cells in 1 mL ice-cold PBS.
6. Add 1 µg of APO1-3 Fas monoclonal antibody except in one condition (*see* **Note 2**).
7. Incubate 5 min at +4 °C.
8. Centrifuge cells at $400 \times g$ for 5 min at +4 °C discard supernatant.

3.3 Lysis

1. Add 1 mL of ice-cold lysis buffer on the cell pellet. On ice, pipet gently up and down a few times to ensure a good lysis.
2. Transfer lysate to a fresh ice-cold 1.5 mL tube.
3. Incubate on ice for 30 min.
4. Centrifuge the lysate at $21,500 \times g$ for 10 min at +4 °C.
5. Transfer supernatant to a fresh ice-cold 1.5 mL tube.

Save 50 μ L of lysate as a per-IP negative control and freeze immediately.

6. Add the Protein AG magnetic beads (*see Note 3*).
7. Incubate for 2 h at 4 °C on a rotating wheel.

3.4 Purification

1. Spin down tubes briefly to remove any droplet from the tubes caps.
2. Place tubes on magnet holder at +4 °C, wait for the magnet to attract beads and for the liquid to clear (approximately 5 min). Visually inspect that beads are on the tube wall before proceeding to the next step.
3. Carefully remove supernatant with a micropipette.
4. Remove tube from the magnet and resuspend beads in 1 mL of fresh ice-cold lysis buffer.
5. Gently invert tubes five times to mix the beads.
6. Repeat **steps 2–6** five times.
7. Centrifuge at $400\times g$ for 30 s at +4 °C.
8. Resuspend sample in 60 μ L of 5 \times sample buffer.
9. Samples can be frozen at –20 °C before proceeding to immunoblotting.

3.5 Immunoblotting of DISC Associated Proteins

1. Component of DISC (CD95/FADD/caspase-8/c-FLIP) can be detected by western-blot analysis.
2. Prepare 12% denaturing polyacrylamide gels to evaluate the amount of proteins present in immunoprecipitation samples and whole lysate controls (*see Note 4*). Since FADD, Caspase-8 and CD95 share bands with similar molecular weight, these molecules have to be analyzed using separate gels.
3. Mount the gels onto the migration assembly and pour the migration buffer into the tank and between the gel plates. Make sure the buffer level is correct.
4. Thaw IP-samples and IP negative samples on ice. When thawed, add 12.5 μ L of 5 \times sample buffer to the IP-negative samples.
5. Denature sample at +95 °C for 5 min in a dry bath before immunoblotting.
6. Load 20 μ L of IP sample and 20 μ L of whole lysate on 12 % SDS polyacrylamide gels. Add a molecular weight ladder.
7. Migrate for 1 h at 150 mA (max 200 V).
8. Transfer proteins onto a nitrocellulose membrane using a semidry system at 0.08 mA/cm² for 2 h.
9. Check transfer efficiency by coloring membrane in ponceau red, then wash ponceau red by rinsing with TBS-T.

Table 1
Recommended antibody concentrations for DISC associated proteins immunodetection

Target	Primary antibody	Recommended dilution	Secondary antibody	Recommended dilution
CD95	CD95 rabbit polyclonal (C-20)	1:5000	Goat anti-rabbit polyclonal	1:2500
Caspase-8	Caspase-8 mouse IgG2b (5F7)	1:5000	Goat anti-mouse IgG2b	1:4000
c-FLIP	c-FLIP mouse IgG1 (7F10)	1:5000	Goat anti-mouse IgG1	1:4000
FADD	FADD mouse IgG1 (A66-2)	1:2000	Goat anti-mouse IgG1	1:4000

10. Saturate the membrane with 5 % dry milk TBST solution for 30 min at room temperature.
11. Wash the blocked membrane with TBS-T.
12. Incubate the membrane with the primary antibody diluted in 5 % dry milk TBS-T solution using the recommended dilutions (Table 1).
13. Wash the nitrocellulose membrane with TBS-T for 15 min, repeat three times.
14. Incubate the membrane with the secondary antibody in 5 % dry milk TBS-T for 60 min at RT.
15. Wash the nitrocellulose membrane with TBS-T for 15 min, repeat three times.
16. Incubate the membrane with ECL revelBlot Intense HRP substrate for 1 min (Ozyme, St Quentin-en-Yvelines, France).
17. Detect luminescence using a light sensitive film or a luminescence imager (Fig. 2).

4 Notes

1. To ensure a better cooling of the tubes, add cold water to the shredded ice.
2. To evaluate the amount of protein off interest that will non specifically “stick” to the magnetic beads, it is important to save one aliquot of unstimulated cells for a “bead only” control condition. This step is performed exactly the same as the other conditions except for the IP antibody that is not added.

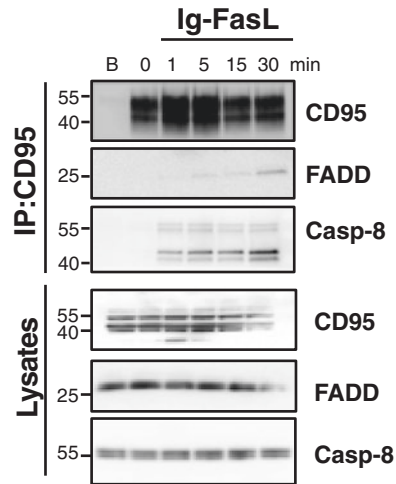


Fig. 2 Immunoblot analysis of the Ig-CD95L-induced DISC. Activated PBLs were stimulated with Ig-CD95L (100 ng/mL) for indicated times, cells were lysed and CD95 was immunoprecipitated. The immune complex was resolved by SDS-PAGE, and the indicated western blots were performed. Total lysates were loaded as a control

3. Protein AG magnetic beads are stored in storage solution. Before use, wash the beads twice with 1 mL lysis buffer without inhibitors and centrifuge for 1 min at $200 \times g$. Repeat once. Then resuspend the beads at the recommended working concentration in lysis buffer with inhibitors added.
4. 10 % polyacrylamide gel preparation:
 - (a) Assemble the two glass plates in the casting system and make sure the assembly is leak-proof by pouring water between the two glass plates. When sure no leak is detectable remove water and dry the plates by inserting a non-plush wiper between the plates. Estimate the desired resolving gel height by inserting the comb and add 1 cm to the bottom of the comb. Mark the height on the plate.
 - (b) Prepare the resolving gel as follow for 1 gel (16 cm \times 7 cm \times 1.5 mm): mix 8.6 mL water, 6 mL of 40 % acrylamide, 5 mL of resolving gel buffer, 200 μ L of 10 % SDS solution, 160 μ L of APS, and 20 μ L of TEMED. Mix gently to avoid excessive bubbles. Pour the mix into the gel cassette. To ensure a plane interface, cover the gel with isopropanol during polymerization. Save the excess resolving gel preparation to monitor polymerization.
 - (c) When polymerized, eliminate isopropanol and rinse with distilled water.

- (d) Prepare the stacking gel as follow: mix 5.8 mL water, 1.5 mL of 40 % acrylamide, 2.5 mL of resolving gel buffer, 100 μ L of 10 % SDS solution, 100 μ L of APS, and 16 μ L of TEMED. Pour the mix on top of the resolving gel and insert a gel comb immediately without introducing air bubbles.

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