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Letter to the Editor

mAb 33 from Transduction Laboratories specifically binds to human CD95-L

Dear Editor,

Deregulated CD95-ligand (CD95-L/Fas-L/APO1-L) protein expression is suggested to be a major pathological mechanism underlying several disorders. Many of these CD95-L alterations were described based on the use of mAb 33 from Transduction Laboratories (Lexington, KY, USA). Since the specifity of this antibody is unclear,[1–3] we have established 293 cells stably transfected with vector encoding the full length human CD95-L cDNA. Overexpression of CD95-L mRNA was ensured by RT–PCR[4] (Figure 1A). Soluble CD95-L in supernatant of these cells induced death in target cells which was prevented by the CD95-L neutralizing mAb NOK-1 (Pharmingen, San Diego, CA, USA) or by blocking $F(ab)_2$ anti-CD95 antibody fragments[5] (Figure 1B). Using Western blotting,[4] mAb 33 and other commercially available CD95-L monoclonal mouse IgG1 antibodies such as G247-4 (Pharmingen, San Diego, CA, USA) and NOK-1 yielded a band at the appropriate size of 37 kD which was increased in CD95-L-transfected cells (Figure 1C). This suggests that CD95-L protein was specifically recognized by G247-4, NOK-1 and mAb 33. However, while mAb 33 produced a single band, several bands were visible using G247-4 or NOK-1 which might be due to glycosylated forms[6] or a high background binding. Another difference between these antibodies is their affinity to CD95-L protein which was highest for mAb 33 (10 μg protein loaded, 10 s exposure time), followed by NOK-1 (10 μg protein loaded, 5 min exposure time) and lowest for G247-4 (50 μg protein loaded, 45 min exposure time). Therefore, mAb 33 is highly sensitive in a

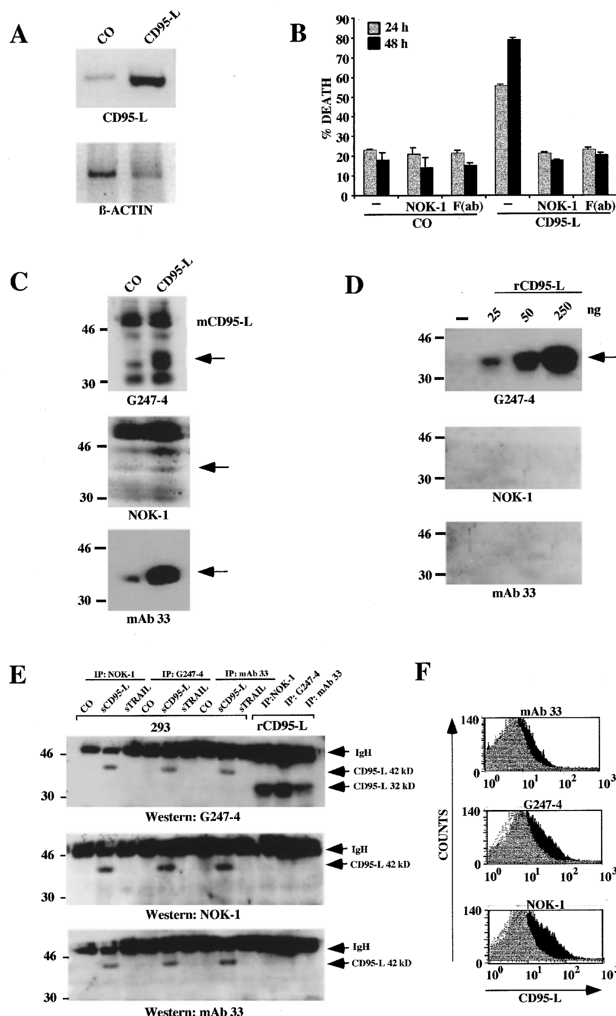

Figure 1 Analysis of CD95-L by RT–PCR, Western blot and Supernatant-transfer-assay (**A**) 293 cells stably transfected with vector encoding the cDNA for full length CD95-L (CD95-L) or empty vector (CO) were examined by RT–PCR for expression of the CD95-L and the β-ACTIN gene. Diminished β-ACTIN amounts in CD95-L overexpressing cells reflect the high spontaneous apoptosis rate in these cells. (**B**) Supernatant form 293 cells was added in a 1 : 1 ratio to JURKAT cells in the presence or absence of NOK-1 (25 μg/ml) or F(ab)2 fragment (1 μg/ml). Death was determined 24 h (grey bars) or 48 h (black bars) later by FACS using FSC/SSC analysis. (**C**) Protein extracts from 293 cells were examined by Western blot using G247-4, NOK-1 or mAb 33. The sizes of a molecular weight marker in kD are indicated on the left. The predicted size of 37 kD for CD95-L is marked by arrows. Ponceau red staining confirmed equal protein loading. (**D**) Recombinant CD95-L protein (rCD95-L) from Alexis was detected by Western blot analysis. The predicted size of 32 to 35 kD for rCD95-L is marked by an arrow. (**E**) For immunoprecipitation of CD95-L supernatants from 293 cells stably overexpressing empty vector or the cDNAs for CD95-L or TRAIL as well as rCD95-L (Alexis) diluted in medium were analyzed by immunoprecipitation with either NOK-1, G247-4 or mAb 33 together with protein A sepharose. Equal parts of each immunoprecipitate were examined in three different Western blot experiments using either G247-4, NOK-1 or mAb 33. The specific bands for soluble CD95-L or the heavy chain of the antibody (IgH) are marked by arrows. The size of a molecular weight marker in kD is indicated on the left. (**F**) Surface expression of CD95-L protein in stably transfected 293 cells was determined using mAb 33, G247-4, NOK-1 or isotype IgG1 control mAb by FACS-analysis and a representative out of five experiments is shown. CD95-L staining is represented by black profiles, empty vector staining by grey profiles and staining controls are marked by dotted lines. Prior to measurement of surface CD95-L expression by FACS-analysis cells were incubated for 12 h with 10^{-3} mM of the CD95-L specific metalloproteinase inhibitor KB8401 (Pharmingen, Hamburg, Germany). After harvesting, cells were stained with 10 μg/ml of either mAb 33, G247-4, NOK-1 or IgG1 isotype control mAb (Becton Dickinson, Heidelberg, Germany) followed by labeling with 20 μg/ml PE-coupled goat anti-mouse secondary Ab (Immunotech, Hamburg, Germany). Fixed cells were analyzed on a FACScan flow cytometer using the Cell Quest software (Becton Dickinson). An ELISA plate coated with immunogens at 2 μg/ml was incubated with dilutions of mAb 33 starting at 1 μg/ml using a standard protocol

Western blot assay and loading too much protein extract may saturate the ECL-reaction masking differences between low or high level expression of CD95-L.[1,2] To further characterize G247-4, NOK-1 and mAb 33, the binding properties to recombinant CD95-L (rCD95-L) from Alexis (Grünberg, Germany) were evaluated. rCD95-L corresponds to the extracellular domain of human CD95-L and is fused at the N-terminus to a linker peptide and a tag. After SDS–PAGE and Western blotting, a specific band with a proposed molecular weight of 32 to 35 kD was detected by G247-4 (Figure 1D). Neither NOK-1 nor mAb 33 stained rCD95-L even after 1 h of exposure following the ECL reaction. On the other hand, NOK-1 completely neutralizes the cytotoxic activity of rCD95-L derived from Alexis (not shown) suggesting that NOK-1 specifically recognizes CD95-L. This means that the reducing conditions of the Western blot experiment together with the linker sequences of the recombinant protein may alter some CD95-L epitopes.

To compare the binding capacities of G247-4, NOK-1 and mAb 33 under non-reducing and reducing conditions we performed an immunoprecipitation assay using supernatants from 293 cells and rCD95-L from Alexis. Supernatants or rCD95-L in cell culture medium were incubated with G247-4, NOK-1 or mAb 33 under non-reducing conditions followed by precipitation with Protein A sepharose (Sigma, Deisenhofen, Germany). The immunoprecipitates were divided into three equal portions and each of them was analyzed by Western blotting. Probing the membranes with either G247-4, NOK-1 or mAb 33 detected enhanced levels of soluble CD95-L protein in CD95-L-transfected 293 cells only (Figure 1E). Soluble CD95-L migrated at a molecular weight of about 42 kD which correspond to a glycosylated form.[6] Thus, full length CD95-L lacking any linker sequences is detected under non-reducing and reducing conditions by G247-4, NOK-1 and mAb 33. Truncated CD95-L protein with linker sequences (Alexis) was also specifically bound by G247-4, NOK-1 or mAb 33 under the non-reducing immunoprecipitation conditions since G247-4 stained a band with the proposed molecular weight. In contrast, neither NOK-1 nor mAb 33 detected immunoprecipitated rCD95-L (Alexis) after Western blotting. These data clearly demonstrate that under reducing conditions linker peptide and tag of rCD95-L mask epitopes which are required for the binding of NOK-1 and mAb 33.

To investigate the functionality of mAb 33, G247-4 and NOK-1 in FACS analysis surface expression of CD95-L in the transfected 293 cells was examined. A species- and isotope-matched antibody was used as control. All of the CD95-L antibodies gave an enhanced staining in CD95-L overexpressing cells although with different affinities since the shift was highest using NOK-1 and lowest using mAb 33 (Figure 1F).

On the basis of these results we conclude that mAb 33 is suited for analyzing natural CD95-L protein by Western blot an immunoprecipitation. mAb 33 is less sensitive in detecting surface expression CD95-L by flow cytometry. mAb 33 specifically binds rCD95-L from Alexis only in its native form e.g. in immunoprecipitation or neutralization assays but not in the denatured form e.g. in Western blot analysis.

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