



Technical note

Quantification of CD95-induced apoptosis and NF- κ B activation at the single cell levelJörn H. Schmidt^a, Sabine Pietkiewicz^a, Michael Naumann^b, Inna N. Lavrik^{a,*}^a Department of Translational Inflammation Research, Institute of Experimental Internal Medicine, Otto von Guericke University, Pfläzler Platz, 39106 Magdeburg, Germany^b Institute of Experimental Internal Medicine, Otto von Guericke University, Leipziger Straße 44, 39120 Magdeburg, Germany

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ABSTRACT

CD95/Fas/APO-1 is a member of the death receptor (DR) family. Stimulation of CD95 leads to the induction of apoptosis as well as to NF- κ B signaling. Crosstalk between these two pathways plays a central role in cell fate. Defects in the regulation of apoptosis and of NF- κ B are connected to a number of chronic inflammatory diseases and cancer. For a better understanding of the life/death decisions in the cell and their contribution to disease progression, the development of new technologies is required. Using imaging flow cytometry we developed a method that enables a quantitative detection of different CD95 signaling pathways in the single cell. The important advantage of this method compared to other approaches is that it allows quantifying a large number of single cells undergoing apoptosis and NF- κ B activation. This technology could provide new insights into the quantitative characterization of apoptosis and NF- κ B at the single cell level and could be used for the quantitative network analysis in systems biology studies.

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1. Introduction

Apoptosis is a programmed type of the cell death common to all multicellular organisms (Krammer et al., 2007; Kaufmann et al., 2012). Apoptosis can be triggered by a number of factors including UV- or γ -irradiation, chemotherapeutic drugs or activation of the death receptors (DRs) (Lavrik and Krammer, 2012). The DR family is a subfamily of the tumor necrosis factor (TNF-R) superfamily (Lavrik et al., 2005a). All members of the DR superfamily are characterized by the presence of the death domain (DD) which plays the central role in transduction of the apoptotic signal (Krammer et al., 2007). In addition to their apoptotic functions, all members of the DR family have been reported to mediate non-apoptotic signaling pathways such as NF- κ B (Neumann et al., 2010; O' Reilly et al., 2009; Strasser et al., 2009).

CD95/Fas/APO-1 is a member of the DR family (Lavrik and Krammer, 2012; Strasser et al., 2009). Stimulation of CD95 with CD95L leads to the induction of both apoptosis and NF- κ B signaling via formation of the death-inducing signaling complex (DISC) (Fig. 1A) (Neumann et al., 2010). This complex serves as a platform for the activation of procaspase-8 followed by activation of effector caspases 3 and 7 and subsequent demolition of the cell (Fig. 1A) (Lavrik et al., 2005b).

The classical NF- κ B activation involves degradation of the NF- κ B bound inhibitors of kappa B (I κ Bs), which are phosphorylated by the

I κ B kinase (IKK)-complex. Consequently, the NF- κ B dimers (p65 and p50) are free to enter the nucleus to activate the transcription of dedicated target genes (Neumann et al., 2007; Neish and Naumann, 2010). CD95-mediated NF- κ B activation has recently been reported to be mediated via c-FLIP proteins and undergo classical NF- κ B activation which involves translocation of the transcription factor p65 to the nucleus (Fig. 1A) (Neumann et al., 2010; Pforr et al., 2011).

Increasing evidence indicates that CD95-mediated apoptosis and NF- κ B occur in parallel in one cell (Neumann et al., 2010). The latter conclusion was based on confocal analysis, and was not supported by quantification. In contrary, there are reports that support the induction of these two pathways in distinct cells (Cullen et al., 2013) Thus, there is a strong urge to develop new methods that allow the investigation of apoptosis and NF- κ B signaling at the single cell level in a quantitative manner. In this study we have developed a method that enables quantitative detection of the two opposing CD95 signaling pathways in single cells using imaging flow cytometry. Importantly, we demonstrate that CD95 stimulation leads to a congruent activation of NF- κ B and apoptosis, and we provide a quantitative characterization of the two pathways.

2. Materials and methods

2.1. Cell culture and stimulation

HeLa cells overexpressing CD95 (HeLa-CD95) were cultivated at 37 °C and 5% CO₂ in DMEM/HamsF12 media (Biochrom, Berlin, Germany) with 10% heat-inactivated FCS (Life Technologies, Darmstadt, Germany), 1%

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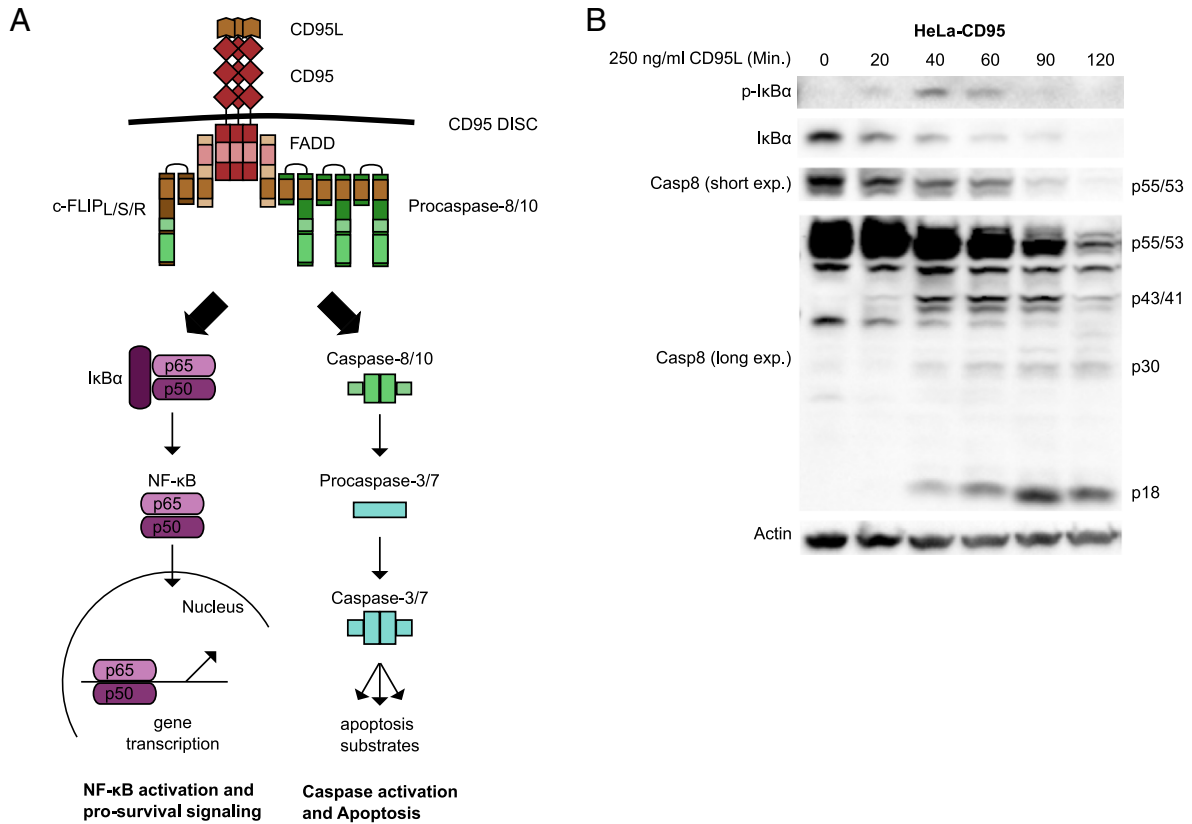


Fig. 1. Monitoring of CD95-mediated NF-κB and apoptosis pathways using Western blot. (A) A scheme of CD95-mediated pro- and anti-apoptotic signal transduction. (B) HeLa-CD95 cells were stimulated with 250 ng/ml of CD95L for indicated periods of time, and subjected to Western blot analysis with the indicated antibodies. One representative Western blot out of three independent experiments is shown.

Penicillin/Streptomycin (Merck Millipore, Darmstadt, Germany) and 10 ng/ml Puromycin (Sigma Aldrich, Taufkirchen, Germany). 10^6 cells were stimulated with indicated concentrations of CD95L (Fricker et al., 2010) for indicated periods of time.

2.2. Sample preparation and staining

After stimulation, cells were washed with ice-cold PBS, detached with Trypsin (GE, Frankfurt a.M., Germany), fixed with 3% formaldehyde in PBS

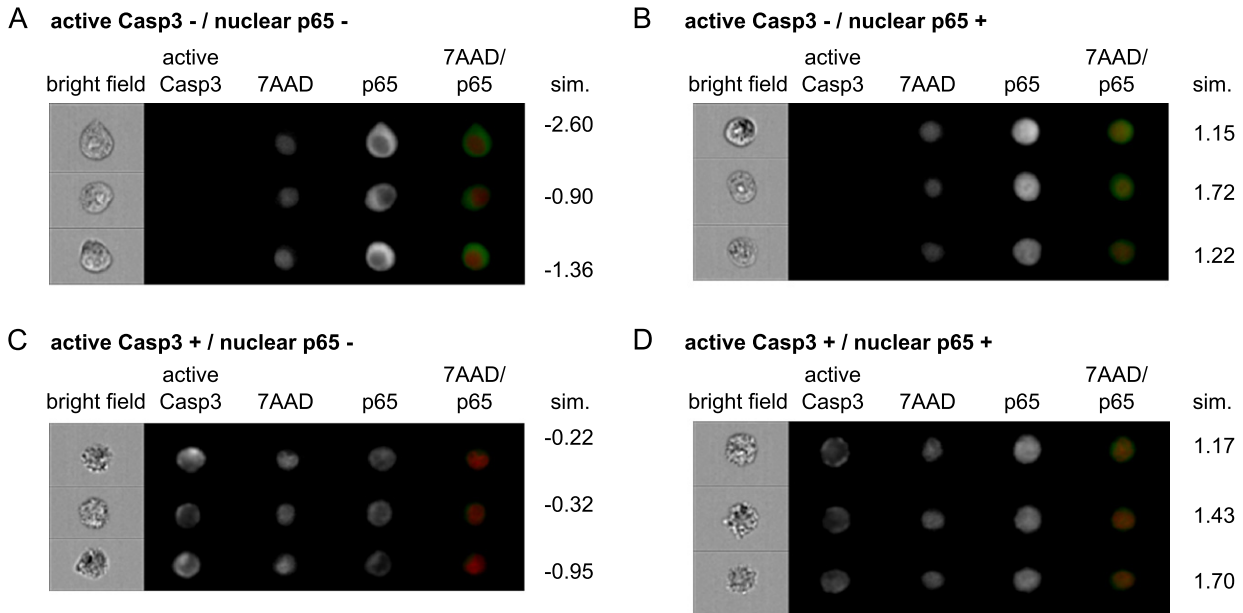


Fig. 2. Monitoring CD95-induced caspase-3 activation and p65 translocation to the nucleus using Imaging Flow Cytometry. (A)–(D). HeLa-CD95 cells were stimulated with 250 ng/ml of CD95L for 60 min. Then the cells were fixed, permeabilized and subjected to staining with anti-cleaved caspase-3-AlexaFluor647, anti-p65-PE antibodies as well as nuclear 7AAD staining. Focused single cells that are double positive for 7AAD and p65-PE were selected. Four populations of the cells are presented with three representative cells per population. Shown are: bright field images, channels 3 (p65), 5 (7AAD) and 11 (active Casp3) and an overlay of channels 3 and 5. Numbers at the right side indicate the similarity morphology (sim) of the 7AAD and p65-PE signals. Active Casp3 –: active Caspase-3 negative, active Casp3 +: active Caspase-3 positive, nuclear p65 –: no nuclear p65, nuclear p65 +: p65 translocated to nucleus.

(Carl Roth, Karlsruhe, Germany) for 10 min at room temperature and permeabilized in 90% ice-cold methanol (Carl Roth, Karlsruhe, Germany) for 30 min on ice. After methanol fixation cells can be stored at -20°C . Before staining cells were washed twice with 1 ml incubation buffer (5 g/l albumin fraction V (AppliChem, Darmstadt, Germany) in PBS (Biochrom, Berlin, Germany)). Cells were stained in 100 μl incubation buffer with 2 μl anti-caspase-3 antibody, conjugated to AlexaFluor647 and recognizing caspase-3 cleaved at Asp175 and 1 μl anti-p65-phycoerythrin (PE) antibody (both antibodies were purchased from Cell Signaling Technologies, Danvers, USA) for 1 h in the dark at room temperature, washed with 1 ml incubation buffer and suspended in 60 μl PBS. At least 5 min before measurement on FlowSight (Amnis/MerckMillipore, Darmstadt, Germany) 3 μl of the DNA-dye 7AAD (BioLegend, San Diego, USA) was added for nuclear staining.

2.3. Data acquisition

The samples were excited with 488 nm and 642 nm lasers, which had an output of 60 mW and 100 mW, respectively. Emission of PE was detected in channel 3 (560 to 595 nm), 7AAD in channel 5 (642 to 745 nm) and AlexaFluor647 in channel 11 (642 to 745 nm). Bright field images were acquired in channel 1 and 9. An automated compensation was performed with samples stained with a single dye. For every sample 10,000 events were recorded.

2.4. Data analysis

Data were analyzed with IDEAS software version 6.0 (Amnis/MerckMillipore, Darmstadt, Germany). First, focused cell pictures were selected by the gradient RMS (gradient root mean square) of the bright

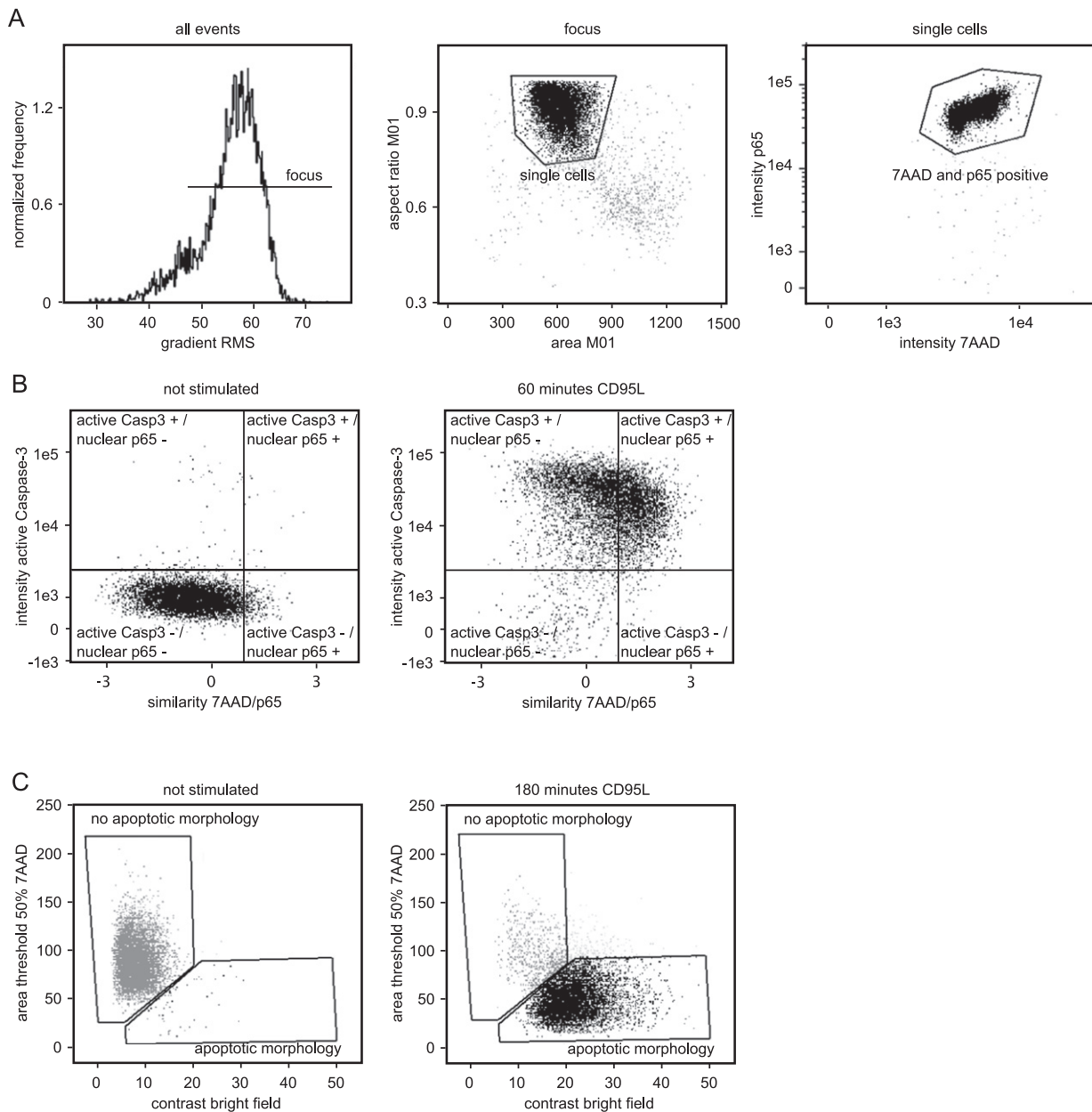


Fig. 3. Gating strategy for quantification of p65 translocation, caspase-3 activation and apoptotic morphology. (A) HeLa-CD95 cells were stimulated with 250 ng/ml CD95L for 60 min, fixed, permeabilized and stained as in Fig. 2. Focused cells were selected by gradient RMS of bright field channel. Single cells were selected by area and aspect ratio of the bright field mask. Only cells that are stained for 7AAD and p65-PE were analyzed further. (B) HeLa-CD95 cells were stimulated for 60 min with 250 ng/ml CD95L to analyze p65 translocation to the nucleus and caspase-3 activation. The similarity morphology of nuclear 7AAD and p65-PE vs. intensity of active Caspase-3 AlexaFluor647 is shown. (C) Contrast of the bright field channel and area threshold 50% of the nuclear channel for focused, single, 7AAD-stained cells without and with stimulation for 180 min with 250 ng/ml CD95L are shown.

field channel and single cells were selected by the area and aspect ratio of the bright field mask as described (Maguire et al., 2011). For the analysis of NF- κ B activation only those cells were selected that were positive for both 7AAD and p65. Following data acquisition, the spatial relationship between the p65 and nuclear images was defined using the “similarity” feature in the IDEAS software (Amnis/ MerckMillipore, Darmstadt, Germany) as previously described (Maguire et al., 2011). This feature allows estimating the degree of correlation between the pixel values of the nuclear 7AAD and the p65-PE signal within a mask surrounding the nuclear 7AAD signal. This parameter was calculated using a log-transformed Pearson’s correlation coefficient (source: Amnis Imaging Flow Cytometer). Accordingly, a low similarity corresponds to a low correlation between two signals and hence indicates a mainly cytoplasmic distribution of p65, while a high similarity exhibits a strong correlation between both signals and hence indicates a nuclear p65 signal. The threshold for the nuclear p65⁺ population was given based on the analysis of the p65 and 7AAD images as described in (Maguire et al., 2011) (Suppl. Fig. 1).

Active caspase-3 was quantified using the fluorescence intensity of the signal from the staining with anti-cleaved-caspase-3 (Asp175) antibodies conjugated to AlexaFluor647. The morphological changes of apoptotic dying cells were quantified by applying the “contrast” and the

“area threshold” features to the analysis of the bright field images of the cell and nucleus. The “contrast” feature describes the sharpness of a picture by detecting changes of pixel values in a 3 × 3 block around every pixel (source: Amnis Imaging Flow Cytometer). The changes in the morphology of the nucleus in the course of apoptosis were analyzed by implementation of the “area threshold” feature to the analysis of the 7AAD images. This can be analyzed by measuring the area of the nuclear signal after applying the area of the “threshold 50%” mask. This mask cuts off the intensity values below the given threshold, i.e. threshold 50% indicates that all the signals below 50% of the maximal intensity value will be excluded. Apoptotic cells show nuclear fragmentation in combination with chromatin condensation and hence show a decreased area of the nucleus with bright signal intensities in comparison to healthy cells. Hence, apoptotic cells have high bright field contrast and low area threshold of the nucleus, while healthy cells can be found with low contrast and high area threshold (Maguire et al., 2011).

2.5. Western blot

Cells were harvested including medium, washed with PBS and lysed for 30 min on ice in lysis buffer (20 mM Tris HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerine, 1% Triton X-100, Protease Inhibitor mix

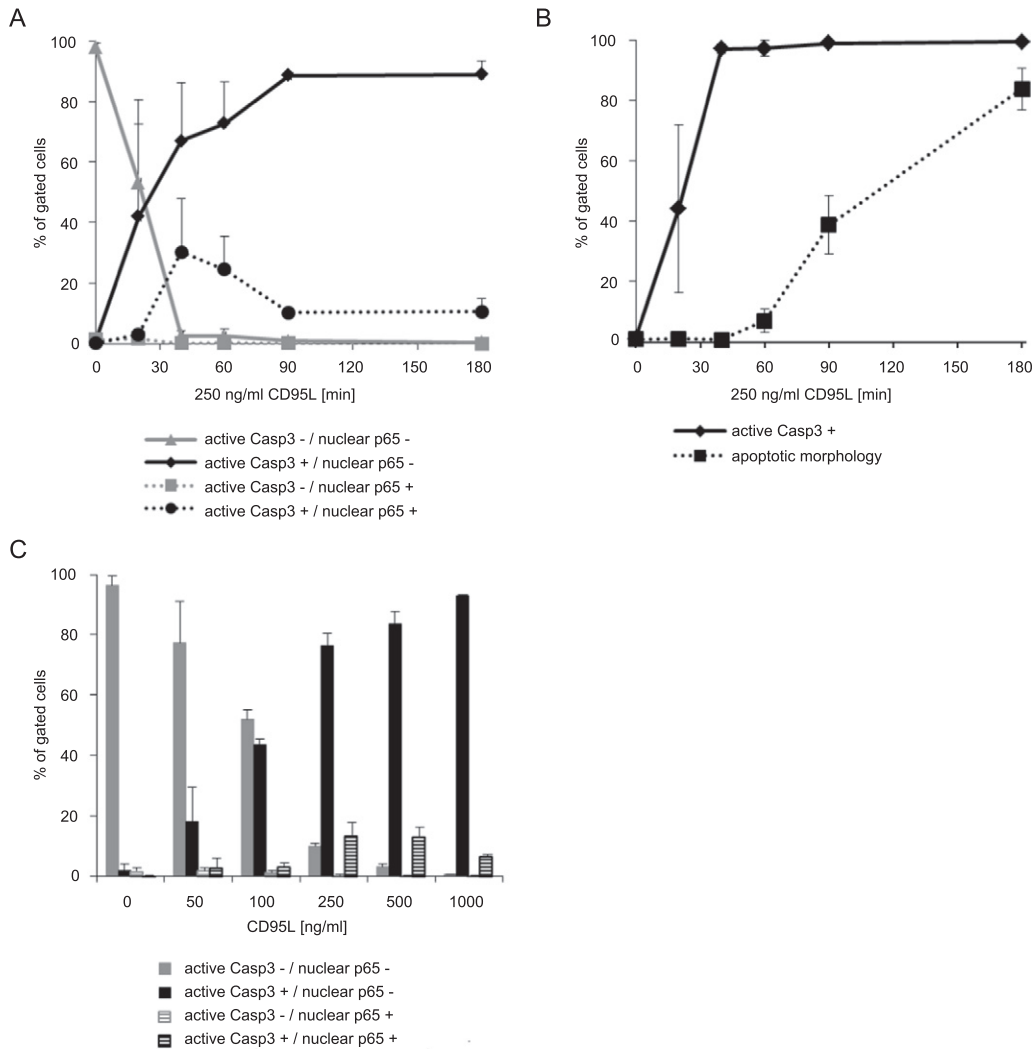


Fig. 4. Quantitative analysis of CD95 signaling. (A) HeLa-CD95 cells were stimulated with 250 ng/ml CD95L for the indicated periods of time. Percentage (%) of gated cells refers to percentage (%) of 7AAD and PE stained cells. (B) Cells from A were analyzed for active caspase-3 and features of apoptotic morphology. Cell gating was done like in Fig. 3C, respectively. (C) HeLa-CD95 cells were stimulated for 40 min with indicated concentrations of CD95L. Active Casp3 - : active Caspase-3 negative, active Casp3 + : active Caspase-3 positive, nuclear p65 - : no nuclear p65, nuclear p65 + : p65 translocated to nucleus. Mean and standard deviation from two to three (A), (B) and three (C) experiments with 10,000 analyzed events per sample are shown.

(Roche, Mannheim, Germany)) and subjected to Western blot. SDS-Page was done with precast 12% stain-free gels. SDS gels were blotted with the TransBlot Turbo system to nitrocellulose membranes (all Biorad, Hercules, USA). Blots were blocked in 5% non-fat dried milk in PBS with 0,05% Tween 20 for one hour, washed three times with PBS-Tween, incubated over night with primary antibodies, washed four times with PBS-Tween and incubated 1 h with HRP-coupled isotype specific secondary antibodies (SantaCruz, Dallas, USA). After washing three times with PBS-Tween, a chemiluminescence signal was produced with LuminataForte (MerckMillipore, Darmstadt, Germany) and detected with a ChemiDoc imaging system (Biorad, Hercules, USA). Anti-Phospho-I κ B- α and anti-I κ B- α antibodies were purchased from Cell Signaling Technologies (Danvers, USA), and anti-Actin from Sigma-Aldrich (Taufkirchen, Germany). The anti-caspase-8 monoclonal antibody C15 (mouse IgG2b), recognizing the p18 subunit of caspase-8, was a kind gift of P. Krammer, DKFZ, Heidelberg. All chemicals were of analytical grade and purchased from CarlRoth (Karlsruhe, Germany), SigmaAldrich (Taufkirchen, Germany) and AppliChem (Darmstadt, Germany).

3. Results and discussion

Stimulation of CD95 with CD95L has been reported to induce both apoptotic and NF- κ B pathways (Neumann et al., 2010). Indeed, using Western blot analysis we observed that stimulation of HeLa-CD95 cells with 250 ng/ml of CD95L resulted in the phosphorylation of I κ B α , its subsequent degradation and procaspase-8 activation (Fig. 1B). The latter was accompanied by the generation of the procaspase-8 cleavage products p43/p41, p30 and p18. Phosphorylation of I κ B α peaked at 40 min after stimulation. Thus, in accordance with previous reports, we detected CD95-mediated activation of caspases and degradation of I κ B α in HeLa-CD95 cells, indicating apoptosis and NF- κ B induction, respectively. However, Western blot analysis does not provide the possibility to follow these events at the single cell level and distinguish whether caspase activation or NF- κ B induction occurs in the same cell or in distinct cells. To address this question we aimed to make use of imaging flow cytometry.

To analyze apoptosis and NF- κ B signaling at the single cell level, we treated HeLa-CD95 cells with 250 ng/ml of CD95L for 60 min. Then the cells were fixed, permeabilized, stained and subjected to imaging flow cytometry analysis. We used three dyes: staining for active caspase-3 (casp3) was performed with anti-caspase-3 antibodies conjugated to AlexaFluor647 and recognizing caspase-3 cleaved at Asp175. This allows monitoring caspase-3 activation in a particular cell. To analyze CD95-mediated NF- κ B activation we followed p65 translocation to the nucleus. The latter approach is based on a combination of anti-p65-PE antibodies and nuclear staining as described (Maguire et al., 2011). We used 7AAD for nuclear staining and visualized p65 translocation to the nucleus (nuclear p65) using the overlay of 7AAD and p65 signals (Fig. 2). Four populations of HeLa-CD95 cells were detected after stimulation with CD95L (Fig. 2). In the first population neither caspase-3 nor p65 translocation to the nucleus was detected (double negative casp3⁻/nuclear p65⁻) (Fig. 2A). The second population, which was rather small, included only cells with p65-translocation to the nucleus (single positive casp3⁻/nuclear p65⁺) (Fig. 2B). The third population comprised cells characterized by caspase-3 staining only (single positive casp3⁺/nuclear p65⁻) (Fig. 2C). Finally, the fourth population consisted of cells that were characterized by both active caspase-3 generation and nuclear p65 (double positive casp3⁺/nuclear p65⁺) (Fig. 2D). Additionally, we were able to follow the changes towards apoptotic morphology using the bright field channel (Fig. 2). Indeed, some of the cells were shrunken and formation of the typical apoptotic blebs was observed. Importantly, in all cells with apoptotic morphology, activation of caspase-3 was detected (Fig. 2). Thus, this analysis demonstrates that CD95 stimulation leads to p65 nuclear translocation and apoptosis in

parallel, giving rise to four populations of cells: casp3⁻/nuclear p65⁻, casp3⁻/nuclear p65⁺, casp3⁺/nuclear p65⁻, and casp3⁺/nuclear p65⁺.

To get a quantitative insight into the ratio between these four populations over time we selected the following gating strategy. Focused single cells were selected (Maguire et al., 2011) and only cells that showed double staining for 7AAD and p65-PE were taken for further analysis (Fig. 3A). The overlay of p65-PE and nuclear 7AAD signals was used to quantify nuclear translocation of p65 (Section 2.4, Fig. 3B). Furthermore, a threshold value (see Section 2) has been used to quantify the signal for p65 translocation, thereby the cells with low changes in the level of p65 translocation to the nucleus were not taken into consideration as nuclear p65⁺ population (Suppl. Fig. 1). Caspase-3 activation was quantified based on the fluorescence intensity of the caspase-3-AlexaFluor647 signal (Fig. 3B). Using this gating strategy we observed that upon stimulation with CD95L for 60 min the cells could be assigned to two major populations: single positive casp3⁺/nuclear p65⁻ and double positive casp3⁺/nuclear p65⁺ cells (Fig. 3B). Interestingly, we did not observe any increase in the population of single positive nuclear p65 cells (casp3⁻/nuclear p65⁺). To assess the apoptotic morphology we implemented "area threshold mask" and "contrast bright field" features of the IDEAS software, which could be used for the analysis of the apoptotic morphology of the dying cells (Section 2.4). Apoptotic cells are characterized by nuclear fragmentation and chromatin condensation and hence show a decreased nuclear area with bright signal intensities in comparison to healthy cells. This can be analyzed by measuring the area of the nuclear signal after applying the "area threshold 50%" mask (Section 2.4). Morphological changes of the membrane (blebbing) in apoptotic dying cells result in a high contrast of the bright field images. Hence, apoptotic cells have high bright field contrast and low area threshold of the nucleus and can be clearly distinguished from normal healthy cells (Fig. 3C).

To get a quantitative insight into the ratio between the four populations over time we stimulated HeLa-CD95 cells for 0, 20, 40, 60, 90 and 180 min with 250 ng/ml of CD95L (Fig. 4A). Without stimulation the majority of the cells belonged to the double negative casp3⁻/nuclear p65⁻ population as they neither showed p65 translocation to the nucleus nor active caspase-3 staining, indicating the absence of apoptosis and p65 activation (Fig. 4A). Upon CD95 stimulation for 20 min, a number of single positive casp3⁺/nuclear p65⁻ cells were already detected indicating active caspase-3 generation within minutes after CD95 engagement. This population increases over time peaking at 90 min. No further increase was observed after 90 min, indicating that most of the cells had undergone cell death afterwards. After 40 min, in addition to a high number of single positive casp3⁺/nuclear p65⁻ cells, the population of double positive casp3⁺/nuclear p65⁺ cells had increased, indicating both active caspase-3 generation and p65 translocation to the nucleus in these cells. This population peaked at 40 min and decreased afterwards, indicating that these cells likely started to undergo cell death. The latter, in turn, leads to changes in nuclear morphology with no detection of nuclear p65. Interestingly, similar to the data presented in Fig. 3B, in this time course we did not observe any increase in the population of single positive nuclear p65 cells (casp3⁻/nuclear p65⁺). This strongly suggests that in response to CD95 stimulation, p65 activation does not take place without caspase activation. Taken together, quantification of the CD95 response in HeLa-CD95 cells over time demonstrates that there are two major populations of cells observed upon CD95 stimulation: single positive casp3⁺/nuclear p65⁻ and double positive casp3⁺/nuclear p65⁺ cells.

To validate whether the cells undergo apoptosis we implemented image analysis of the bright field and nuclear channels. As mentioned above, the apoptotic cells undergo changes in the morphology of the nucleus and the membrane, which can be detected and quantified using the nuclear area threshold mask and the contrast bright field feature (Fig. 3C). Quantification of this process over time demonstrates that caspase-3 activation precedes the apoptotic morphological changes (Fig. 4B). The caspase-3-positive cells in this quantification (Fig. 4B)

comprised both single positive casp3⁺/nuclear p65⁻ and double positive casp3⁺/nuclear p65⁺ cells and, therefore, the number of caspase-3-positive cells was larger as compared to single populations in Fig. 4A. Upon stimulation with 250 ng/ml within one hour we already observed that most of the cells were caspase-3 positive. At this time point only 10% of the cells started to undergo cell death. Interestingly, within the next 30 min, the population of dying cells drastically increased reaching about 40% of the total cellular population. Finally, at 180 min after stimulation there were almost no healthy cells left. This analysis shows that the morphological changes of the cells, e.g. membrane blebbing and fragmentation of the nucleus, are quickly initiated leading to cell death after the cells become caspase-3 positive.

To characterize CD95-mediated signaling in a dose-dependent way we have stimulated HeLa-CD95 cells for 40 min with different concentrations of CD95L (Fig. 4C). The cells underwent dose-dependent caspase-3 activation and p65 translocation to the nucleus (Fig. 4C). Caspase-3 activation increased with the higher stimulation strength. The population corresponding to the double positive caspase-3⁺/nuclear p65⁺ cells was going down upon 1000 ng/ml stimulation indicating that most of the cells had most likely undergone apoptosis at this concentration of CD95L. Furthermore, as mentioned above, we considered only the cells with the higher level of p65 translocation, e.g. above the arbitrary given threshold (Suppl. Fig. 1). Thus, we do not consider the cells with the low signal of p65 translocation to the nucleus, thus, the overall number of cell with a change in nuclear p65 translocation might be higher. Finally, p65 translocation to the nucleus is a very dynamic process (Nelson et al., 2004) therefore the used time point might cover only a section of p65 regulation. These data show, that upon CD95 stimulation the majority of cells undergo apoptosis, which might be accompanied by p65 translocation to the nucleus. Furthermore, importantly, the NF- κ B pathway in CD95 signaling is directly linked to apoptosis and caspase activity, indicating the possibility of similar regulatory molecules mediating these two pathways. The latter is in accordance with previous reports (Neumann et al., 2010). As mentioned above there have been a number of contradictory reports on whether cells that induce NF- κ B can also undergo apoptosis or these pathways do not occur in parallel. Our method unraveled that these pathways take place simultaneously in the same cell. The application of this approach to other cellular settings and monitoring the activation of cell death vs. NF- κ B activation at the single cell level will certainly provide new insights into the molecular mechanisms of DR signaling and pave the road towards the development of new treatments in personalized medicine.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2015.04.026>.

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References

- Cullen, S.P., Henry, C.M., Kearney, C.J., Logue, S.E., Feoktistova, M., Tynan, G.A., Lavelle, E.C., Leverkus, M., Martin, S.J., 2013. Fas/CD95-induced chemokines can serve as “find-me” signals for apoptotic cells. *Mol. Cell* 49, 1034.
- Fricke, N., Beaudouin, J., Richter, P., Eils, R., Krammer, P.H., Lavrik, I.N., 2010. Model-based dissection of CD95 signaling dynamics reveals both a pro- and antiapoptotic role of c-FLIPL. *J. Cell Biol.* 190, 377.
- Kaufmann, T., Strasser, A., Jost, P.J., 2012. Fas death receptor signalling: roles of Bid and XIAP. *Cell Death Differ.* 19, 42.
- Krammer, P.H., Arnold, R., Lavrik, I.N., 2007. Life and death in peripheral T cells. *Nat. Rev. Immunol.* 7, 532.
- Lavrik, I.N., Krammer, P.H., 2012. Regulation of CD95/Fas signaling at the DISC. *Cell Death Differ.* 19, 36.
- Lavrik, I., Golks, A., Krammer, P.H., 2005a. Death receptor signaling. *J. Cell Sci.* 118, 265.
- Lavrik, I.N., Golks, A., Krammer, P.H., 2005b. Caspases: pharmacological manipulation of cell death. *J. Clin. Invest.* 115, 2665.
- Maguire, O., Collins, C., O’Loughlin, K., Miecznikowski, J., Minderman, H., 2011. Quantifying nuclear p65 as a parameter for NF-kappaB activation: correlation between ImageStream cytometry, microscopy, and Western blot. *Cytometry A* 79, 461.
- Neish, A.S., Naumann, M., 2010. Microbial-induced immunomodulation by targeting the NF- κ B system. *Trends Microbiol.* 19, 596.
- Nelson, D.E., Ihekweba, A.E., Elliott, M., Johnson, J.R., Gibney, C.A., Foreman, B.E., Nelson, G., See, V., Horton, C.A., Spiller, D.G., Edwards, S.W., McDowell, H.P., Unitt, J.F., Sullivan, E., Grimley, R., Benson, N., Broomhead, D., Kell, D.B., White, M.R., 2004. Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science* 306, 704.
- Neumann, M., Naumann, M., 2007. Beyond IkappaBs: alternative regulation of NF-kappaB activity. *FASEB J.* 21, 2642.
- Neumann, L., Pforr, C., Beaudouin, J., Pappa, A., Fricke, N., Krammer, P.H., Lavrik, I.N., Eils, R., 2010. Dynamics within the CD95 death-inducing signaling complex decide life and death of cells. *Mol. Syst. Biol.* 6, 352.
- O’Reilly, L.A., Tai, L., Lee, L., Kruse, E.A., Grabow, S., Fairlie, W.D., Haynes, N.M., Tarlinton, D.M., Zhang, J.G., Belz, G.T., Smyth, M.J., Bouillet, P., Robb, L., Strasser, A., 2009. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature* 461, 659.
- Pforr, C., Neumann, L., Eils, R., Krammer, P.H., Lavrik, I.N., 2011. Understanding life and death at CD95. *Adv. Exp. Med. Biol.* 691, 151.
- Strasser, A., Jost, P.J., Nagata, S., 2009. The many roles of FAS receptor signaling in the immune system. *Immunity* 30, 180.

