Short Communication

Intracellular Delivery of Synthetic dsRNA to Leukemic Cells Induces Apoptotic and Necrotic Cell Death

(leukaemia / poly I:C / electroporation / apoptosis)

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Abstract. The type of tumour cell death dictates the type of adaptive immune response mounted against the tumours. In haematological malignancies such as acute myeloid leukaemia (AML), immune evasion due to the poor immunogenicity of leukemic cells is a major hurdle in generating an effective immune response. Transfection of synthetic dsRNA, poly I:C, into leukemic cells to trigger tumour cell death and enhance immunogenicity of the tumour is a promising immunotherapeutic approach. However, the temporal cell death kinetics of poly I:C-electroporated AML cells has not been thoroughly investigated. Electroporation of U937 cells, a human AML cell line, with a high dose of poly I:C resulted in cytotoxicity as early as 1 h post-transfection. Flow cytometric analysis revealed the temporal switch from early apoptosis to late apoptosis/secondary necrosis in poly I:C-electroporated cells in which the nuclear morphology at later time points was consistent with necrotic cell death. Our brief findings demonstrated the temporal cell death kinetics of dsRNA-transfected leukemic cells. This finding is an important development in the field of dsRNA immunotherapy for leukaemia as understanding the type of cell death elicited by transfected dsRNA will dictate the type of immune response to be directed against leukemic cells.

Introduction

Cell death is one of the most fundamental cellular responses, which is tightly regulated to maintain physiological homeostasis and the overall health of an organism. Dysregulation in the cell death mechanism disrupts this balance, resulting in diseases with premature cell loss (e.g. neurodegenerative diseases) or persistent cell survival (e.g. cancer and autoimmune diseases). In terms of cancer, induction of cell death in the tumour is the most attractive strategy for anti-cancer therapy. Depending on the stimuli, tumour cells can die through distinct cell death mechanisms including apoptosis and necrosis. In leukemic diseases such as acute myeloid leukaemia (AML), immune evasion of circulating leukemic cells due to their low to negligible immunogenicity makes establishment of anti-tumour immunity challenging (Curran et al., 2015). Considering the poor efficacy of the current treatment agents for leukaemias, investigation is warranted for novel immunotherapeutic interventions.

A promising immunotherapeutic strategy to boost immunogenicity is intracellular introduction of pathogen-associated molecular patterns such as polyinosinic-polycytidylic acid (poly I:C), a synthetic double-stranded RNA (dsRNA), into leukemic cells, which mimics viral infection. Depending on the cell type, dose of dsRNA, and method of delivery, the cells respond to this "danger" signal by either initiating cell death or a cell activation response (reviewed by Idris, 2014)). The type of tumour cell death is important because it will dictate the efficiency and processing of exogenous tumour antigens by dendritic cells (DCs) (Inzkirweli et al., 2007; Kushwah et al., 2010; Zelenay et al., 2012), antigen-presenting cells of the adaptive immune system. Leukemic cell death induced by poly I:C transfection has been shown to enhance tumour cell immunogenicity (Smits et al., 2007) and dead tumour cell uptake by DCs (Lion et al., 2011). The majority of poly I:C-transfected NB4 cells, a human myeloid leukemic cell line, die through late apoptosis in a poly I:C dose-dependent manner at 24 h post-transfection (Smits et al., 2007). A subsequent study reported similar findings in two other human myeloid leukemic cell lines, K562 and U937, but without conclusively showing that poly I:C electroporation leads to apoptosis (Lion et al., 2011). Therefore, an accurate temporal assessment of the cell death

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Abbreviations: AML – acute myeloid leukaemia, DCs – dendritic cells, dsRNA – double-stranded RNA, PI – propidium iodide, poly I:C – polyinosinic-polycytidylic acid.

responses in poly I:C-dependent cytotoxicity of leukemic cells is necessary.

Therefore, we hypothesise that U937 cells die by apoptosis at early time points and by necrosis at later time points post-transfection with poly I:C. In this study, we show that poly I:C-electroporated U937 cells die in a time- and dose-dependent manner. Furthermore, poly I:C-electroporated U937 cells die through apoptosis as early as 6 h post-transfection before switching to cell death-resembling necrosis at 24 h.

Material and Methods

Cell culture

The human AML cell line U937 was obtained from the American Tissue Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10 % foetal calf serum (Invitrogen), 2 mM L-glutamine, 20 U/ml penicillin, and 20 µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Electroporation of poly I:C into U937 cells

Prior to electroporation, 4×10^6 cells/ml in medium were mixed with HEPES buffer (pH 7.0) to a final concentration of 10 mM. Cells (250 µl containing 1×10^6 cells) were transferred into 4-mm gap electroporation cuvettes (Bio-Rad, Hercules, CA) together with various amounts of poly I:C (Sigma, St. Louis, MO) that were previously prepared in sterile nuclease-free water. Sterile nuclease-free water without poly I:C was used as a negative control for transfection. Electroporation was performed at 240 V with a capacitance of 975 µF at room temperature in a Gene Pulser XCell™ (Bio-Rad), which is similar to conditions in previous studies (Roberts et al., 2009; Sagulenko et al., 2013).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

After electroporation, the electroporated cells and control cells were seeded in a 96-well tissue culture plate (Corning, Tewksbury, MA) at 1×10^5 cells/well in quadruplicate for each type of treatment conditions and incubated at 37 °C. The MTT assay was performed after appropriate incubation times by addition of 10 µl of 5 mg/ml MTT (Sigma) to each well, followed by 4 h of incubation. The plate was then centrifuged at 700 *g* for 15 min before removing the supernatant. DMSO (200 µl) (Sigma) was then added to the cell pellet, followed by incubation in the dark overnight. The next day, the absorbance of the wells at 630 nm was analysed spectrophotometrically in a BioTek ELx808 ELISA plate reader (BioTek, Winooski, VT).

Lactate dehydrogenase (LDH) assay

After electroporation, the electroporated cells and control cells were seeded in a 96-well tissue culture plate (Corning) at 1×10^5 cells/well in quadruplicate for each treatment type of conditions and incubated at 37 °C. At the indicated time points, the plate was centrifuged at 700 *g* for 15 min before removing the supernatant. LDH release determinations were performed using fresh culture supernatants and a Cytotoxicity Detection kit (Sigma) according to the manufacturer's instructions. Triton X-100 (2%) was added to untreated cells as a positive control. Absorbance was measured at 490 nm in the BioTek ELx808 ELISA plate reader (BioTek).

Cytospinning and nuclear staining

Cells were harvested and fixed in 4% paraformaldehyde (Sigma) for 30 min at room temperature. The cells were then centrifuged at 700 *g* for 5 min and washed three times in PBS. The cells were resuspended in PBS at a density of 1×10^6 cells/ml and cytospun on 25- × 75- × 1.0-mm electrostatically Superfrost Plus Gerhard Menzel charged glass slides (Thermo Scientific, Waltham, MA) at 700 rpm for 5 min with medium acceleration in a Thermo Shandon Cytospin 4 cytospinner (Thermo Scientific). The cells on the slides were rehydrated by washing in PBS and then stained with 50 ng/ml 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Invitrogen) in PBS for 10 min. After staining, the cells were washed three times in PBS, mounted with Dako-Cytomation florescence mounting medium (Dako, Glostrup, Denmark) before placing coverslips over the cells. The stained cells were viewed under an epi-fluorescence microscope at 100× magnification.

Annexin V/propidium iodide (PI) staining

After electroporation, both control and poly I:C-electroporated cells were cultured in complete medium in 35-mm cell culture dishes (Corning) at 1×10^6 cells/dish in a humidified incubator at 37 °C. After 6 and 24 h, the cell suspension was centrifuged and the supernatant was discarded. The cells were then stained using an Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's instructions and analysed in a BD Accuri® C6 Flow Cytometer (BD Bioscience, San Jose, CA).

Results and Discussion

We used U937 cells in this study as a model cell line for AML. Previous studies have introduced nucleic acids, including oligodeoxynucleotides (Bergan et al., 1996; Shimokawa et al., 2000) and poly I:C (Lion et al., 2009, 2011; Smits et al., 2007), into U937 cells by electroporation. Compared with other transfection methods, electroporation appears to deliver macromolecules directly into the cytosol (Sun et al., 2014), avoiding endocytic pathways and associated nucleases (Bamford et al., 2014). Artificial introduction of poly I:C into the cytosolic space closely resembles viral infection. Although a previous study showed that electroporation of U937 cells with poly I:C results in cell death after 24 h, the

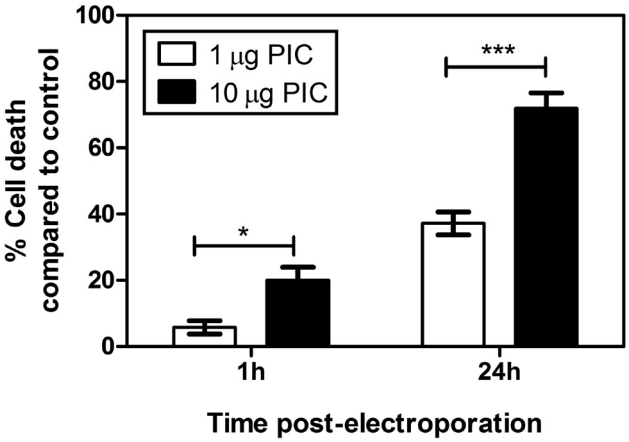

Fig. 1. Poly I:C-electroporated U937 cells die in a time- and dose-dependent manner.
U937 cells were electroporated with or without the indicated doses of poly I:C (PIC), incubated at 37 °C to the indicated time points, and then subjected to MTT assays. The percentage of cell death was determined by comparing the effects of electroporation with poly I:C or without poly I:C (Control). Data represent the mean ± SEM of at least four independent experiments. *P < 0.05, ***P < 0.0001, Student's *t*-test

exact mode of cell death was not examined (Lion et al., 2011).

To assess the effects of various doses of poly I:C on U937 cell viability over time, the cells were electroporated with or without various doses of poly I:C and then analysed by MTT assay, a colorimetric assay that measures the metabolic activity of cells, at various time points. Compared with electroporation alone, the percentage of cell death was higher using 10 μg poly I:C compared with 1 μg poly I:C as early as 1 h post-electroporation (Fig. 1). This result demonstrated that poly I:C electroporation-mediated loss of viability in U937 cells is dependent on the poly I:C dose and time post-electroporation, which is consistent with previous findings in NB4 cells (Smits et al., 2007). In future studies, it would be interesting to investigate the effect of synthetic dsRNA length on the cell death.

Next, we assessed the mode of cell death. Cells undergoing apoptosis typically stain positive for annexin V, a phosphatidylserine that is externalized to the outer cell membrane during apoptosis (Vermes et al., 1995). Non-apoptotic cell death, such as necrosis, allows PI to enter cells because the cell membrane becomes permeable owing to the loss of plasma membrane integrity (Golstein and Kroemer, 2007). Flow cytometric analysis at 24 h post-electroporation revealed that a significant part of non-viable poly I:C-electroporated cells [cells that are not annexin V(-)/PI(-)] were annexin V(+)/PI(+) compared with cells electroporated alone, even at a low dose of poly I:C (1 μg) (Fig. 2). This staining pattern is consistent with secondary necrosis/late apoptosis events. Our observations corroborated previous findings in NB4 cells (Smits et al., 2007), although the dose required to reach the same proportion of cells undergoing secondary necrosis/late apoptosis (~50 %) in our study (1 μg poly I:C) was far less than that required in the other study (20–100 μg poly I:C).

The transfection efficiency based on differences in the electroporation conditions used in our study (240 V; capacitance: 1000 μF) and the other study (300 V; capacitance: 150 μF) could account for this discrepancy. Considering that the conditions used in the previous study resulted in a lower transfection efficiency of poly I:C in another cell line (Schulz et al., 2005), it is likely that our electroporation conditions allowed more poly I:C to enter the cytosol. However, at an earlier time point (6 h), a significant percentage of non-viable cells electroporated with 10 μg poly I:C were annexin V(+)/PI(-), which is consistent with early apoptotic events (Fig. 2). The percentages of annexin V(+)/PI(-)-stained cells that were electroporated alone or with 1 μg poly I:C were comparable. Overall, our data suggest that poly

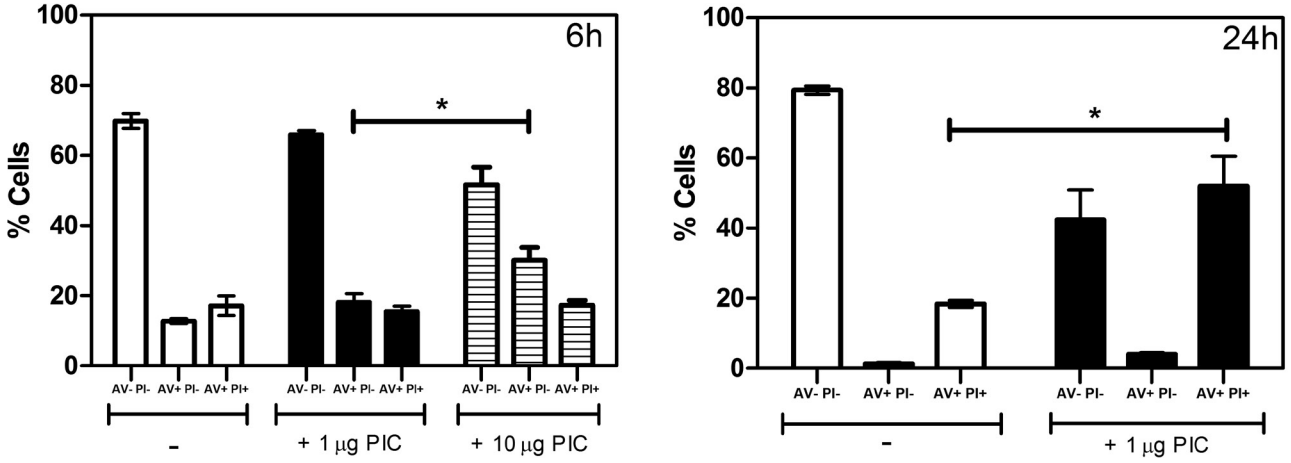

Fig. 2. Poly I:C-dependent cell death at 24 h post-electroporation is consistent with secondary necrosis/late apoptosis.
U937 cells were electroporated with or without the indicated doses of poly I:C (PIC), incubated at 37 °C to the indicated time points, and then stained with annexin V and PI for flow cytometric analysis. Graphs show the mean ± SEM of three independent experiments. *P < 0.05, Student's *t*-test

I:C electroporation into U937 cells triggers early apoptotic events at earlier time points before transitioning to a late apoptotic/secondary necrotic cell death type. However, in future work, a more extensive time course is needed to pinpoint the exact onset of different cell death modalities in response to poly I:C transfection.

To unambiguously discriminate between late apoptotic and necrotic cells (annexin V(+)/PI(+) stained cells) is challenging because there is no clear biochemical definition of necrotic cell death (Golstein and Kroemer, 2007). A key signature for necrotic cells is permeabilization of the plasma membrane. This event can be quantified by measuring the release of intracellular enzyme LDH, a soluble cytoplasmic enzyme that is present in almost all cells and released into the extracellular space when there is destruction of the plasma membrane. Indeed, the loss of plasma membrane permeability was observed in cells electroporated with poly I:C in a dose-dependent manner compared with cells electroporated alone at 24 h post-electroporation (Fig. 3A). Although cells undergoing late apoptosis also exhibit loss of plasma membrane permeability (Gregory and Devitt, 2004), microscopic examination of cells electroporated with a high dose of poly I:C (10 μg) at 24 h post-electroporation revealed nuclear morphological features resembling necrosis rather than late apoptosis (Fig. 3B).

In the late stages of apoptosis, nuclei fragment and condense to form distinct apoptotic bodies (Gregory and Devitt, 2004). It is possible that the reported increase in the immunogenicity of poly I:C-electroporated leukemic cells (Lion et al., 2009, 2011, 2014; Smits et al., 2007) is not solely due to recognition of apoptotic tumour cells, but could potentially be a response to necrotic tumour cells. The decision of antigen-presenting cells, such as DCs, to mount an immunogenic or tolerogenic adaptive immune response against internalized material has strong implications in devising the most appropriate immunotherapeutic treatment for leukemic diseases such as AML. Using dsRNA-transfected AML cells as a vaccine for immunotherapy of leukaemia to initiate the most appropriate form of leukemic cell death is essential. Because late apoptotic and necrotic cells are generally considered activators of the immune system, elucidation of the molecular mechanisms underlying responses to these cells is crucial.

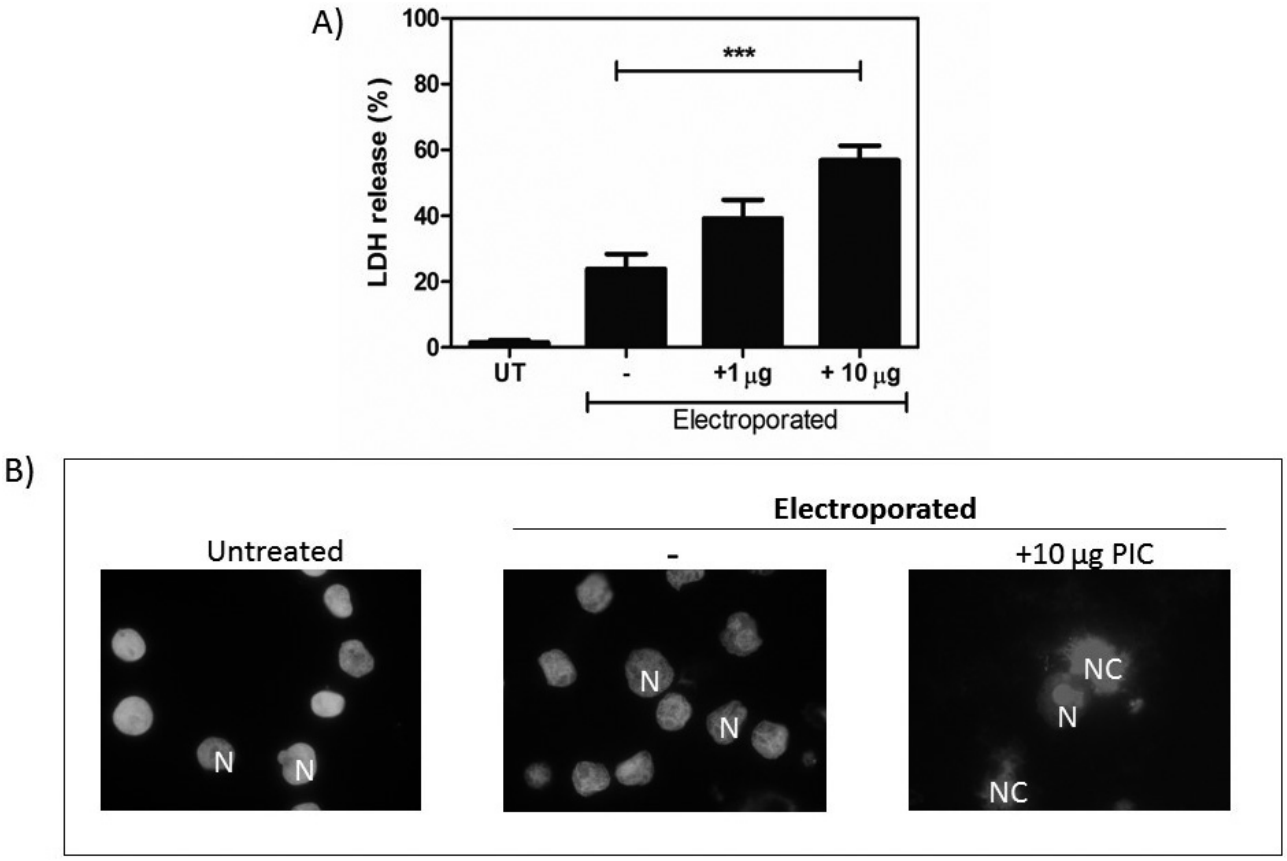

Fig. 3. Loss of plasma membrane permeability is associated with poly I:C-mediated cell death at 24 h post-electroporation and cells exhibit typical characteristics of necrotic cell death.

A) U937 cells were electroporated with or without the indicated doses of poly I:C (PIC), incubated for 24 h at 37 °C, and then analysed for LDH release. Data represent the mean ± SEM of three independent experiments. ***P < 0.0001, Student's *t*-test.

B) Cells were fixed with 4% paraformaldehyde at 24 h post-electroporation with 10 μg poly I:C (PIC). Cell nuclei were stained with DAPI and observed at 100×. N – normal nuclei; NC – necrotic-like nuclei

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Authors disclosure statement

No competing financial interests exist.

References

Bamford, R. A., Zhao, Z.-Y., Hotchin, N. A., Styles, I. B., Nash, G. B., Tucker, J. H., Bicknell, R. (2014) Electroporation and microinjection successfully deliver single-stranded and duplex DNA into live cells as detected by FRET measurements. *PLoS One* **9**, e9597

Bergan, R., Hakim, F., Schwartz, G. N., Kyle, E., Cepada, R., Szabo, J. M., Fowler, D., Gress, R., Neckers, L. (1996) Electroporation of synthetic oligodeoxynucleotides: a novel technique for ex vivo bone marrow purging. *Blood* **88**, 731-741.

Curran, E., Corrales, L., Kline, J. (2015) Targeting the innate immune system as immunotherapy for acute myeloid leukemia. *Front. Oncol.* **5**, 83.

Golstein, P., Kroemer, G. (2007) Cell death by necrosis: towards a molecular definition. *Trends Biochem. Sci.* **32**, 37-43.

Gregory, C. D., Devitt, A. (2004) The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology* **113**, 1-14.

Idris, A. (2014) Cellular responses to cytosolic double-stranded RNA – role of the inflammasome. *Immunol. Immunogenet. Insights* **6**, 7-11.

Inzkirweli, N., Gueckel, B., Sohn, C., Wallwiener, D., Bastert, G., Lindner, M. (2007) Antigen loading of dendritic cells with apoptotic tumor cell-preparations is superior to that using necrotic cells or tumor lysates. *Anticancer Res.* **27**, 2121-2129.

Kushwah, R., Wu, J., Oliver, J. R., Jiang, G., Zhang, J., Siminovitch, K. A., Hu, J. (2010) Uptake of apoptotic DC converts immature DC into tolerogenic DC that induce differentiation of Foxp3+ Treg. *Eur. J. Immunol.* **40**, 1022-1035.

Lion, E., Smits, E. L., Berneman, Z. N., Van Tendeloo, V. F. (2009) Acute myeloid leukemic cell lines loaded with synthetic dsRNA trigger IFN-γ secretion by human NK cells. *Leuk. Res.* **33**, 539-546.

Lion, E., Anguille, S., Berneman, Z. N., Smits, E., Van Tendeloo, V. (2011) Poly (I: C) enhances the susceptibility of leukemic cells to NK cell cytotoxicity and phagocytosis by DC. *PLoS One* **6**, e20952.

Lion, E., de Winde, C. M., Van Tendeloo, V. F., Smits, E. L. (2014) Loading of acute myeloid leukemia cells with poly (I: C) by electroporation. *In: Cancer Vaccines,* eds. M. J. P. Lawman, P. D. Lawman, **1139**, 233-241, Springer, New York.

Roberts, T. L., Idris, A., Dunn, J. A., Kelly, G. M., Burnton, C. M., Hodgson, S., Hardy, L. L., Garceau, V., Sweet, M. J., Ross, I. L., Hume, D. A., Stacey, K. J. (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* **323**, 1057-1060.

Sagulenko, V., Thygesen, S., Sester, D., Idris, A., Cridland, J. A., Vajjhala, P. R., Roberts, T. L., Schroder, K., Vince, J. E., Hill, J. M., Silke, J., Stacey, K. J. (2013) AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ.* **20**, 1149-1160.

Schulz, O., Diebold, S. S., Chen, M., Näslund, T. I., Nolte, M. A., Alexopoulou, L., Azuma, Y. T., Flavell, R. A., Liljeström, P., Reis e Sousa, C. (2005) Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* **433**, 887-892.

Shimokawa, T., Okumura, K., Ra, C. (2000) DNA induces apoptosis in electroporated human promonocytic cell line U937. *Biochem. Biophys. Res. Commun.* **270**, 94-99.

Smits, E., Ponsaerts, P., Van de Velde, A., Van Driessche, A., Cools, N., Lenjou, M., Nijs, G., Van Bockstaele, D. R., Berneman, Z. N., Van Tendeloo, V. F. (2007) Proinflammatory response of human leukemic cells to dsRNA transfection linked to activation of dendritic cells. *Leukemia* **21**, 1691-1699.

Sun, C., Cao, Z., Wu, M., Lu, C. (2014) Intracellular tracking of single native molecules with electroporation-delivered quantum dots. *Anal. Chem.* **86**, 11403-11409.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutellingsperger, C. (1995) A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* **184**, 39-51.

Zelenay, S., Keller, A. M., Whitney, P. G., Schraml, B. U., Deddouche, S., Rogers, N. C., Schulz, O., Sancho, D., Reis e Sousa, C. R. (2012) The dendritic cell receptor DNGR-1 controls endocytic handling of necrotic cell antigens to favor cross-priming of CTLs in virus-infected mice. *J. Clin. Invest.* **122**, 1615-1627.