Dominance of cytokine- over FasL-induced impairment of the mitochondrial transmembrane potential (Δψ\textsubscript{m}) in the pancreatic β-cell line NIT-1

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Abstract

Mitochondria of pancreatic β-cells are potential targets of intrinsic and extrinsic apoptotic pathways in the autoimmune pathogenesis of type 1 diabetes. We aimed to investigate whether cytokine- and FasLigand (FasL)-induced apoptosis is associated with impaired mitochondrial transmembrane potential (Δψ\textsubscript{m}) in the pancreatic β-cell line NIT-1. NIT-1 cells were exposed to the interleukin-1β/interferon-γ (IL-1β/IFN-γ) cytokine combination to induce apoptosis in vitro. Low concentrations of cytokines resulted in Δψ\textsubscript{m} impairment, and increasing concentrations had only a minor additional effect. Treatment with the inducible nitric oxide synthase (iNOS) inhibitor N\textsubscript{o}-nitro-l-arginine methyl ester hydrochloride (L-NAME) prevented cytokine-mediated Δψ\textsubscript{m} impairment, implying that cytokines affect Δψ\textsubscript{m} via nitric oxide. The broad-spectrum caspase inhibitor Z-VAD(Ome)-FMK (ZVAD) revealed dichotomous actions. In the presence of ZVAD, cytokine-induced nitrite generation was increased but cell death and Δψ\textsubscript{m} impairment were reduced. Δψ\textsubscript{m} impairment was also reduced by inhibitors of caspases 1, 6 and 8. Induction of Fas by IL-1β/IFN-γ coupled with activation by Super-FasL augmented cytokine-induced cell death. We observed a clear dominance of cytokine- over FasL-induced effects on Δψ\textsubscript{m}.

Our findings show that IL-1β/IFN-γ cytokines have a strong effect to impair Δψ\textsubscript{m} and prime β-cells for apoptosis via the intrinsic pathway mediated by iNOS and caspases. Furthermore, at least in NIT-1 cells, the extrinsic FasL/Fas pathway has only a minor additive effect on cytokine-induced Δψ\textsubscript{m} impairment.

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Introduction

Apoptosis occurs by two major transduction mechanisms, the extrinsic and intrinsic pathways, and can be activated by a diverse set of external or internal signals.\(^1\) In the extrinsic pathway, apoptosis is induced by the interaction of ligands with receptors belonging to the tumour necrosis factor death-receptor superfAMILY. The most commonly investigated apoptotic induction pathway is the Fas/FasL pathway, in which FasLigand (FasL) induces apoptosis in Fas-expressing cells.

Oxidative stress and DNA damage are potential inducers of apoptosis via the intrinsic pathway,\(^2\) in which mitochondria are the central players.\(^3\) The electrochemical gradient across the inner mitochondrial membrane required for oxidative phosphorylation, Δψ\textsubscript{m}, results from the respiratory-driven, electron transport chain-mediated pumping of protons out of the inner membrane, and is indispensable for driving ATP synthesis.\(^3,4\) Loss of Δψ\textsubscript{m} is considered to be an irreversible step in the apoptotic process and, in addition to other cellular events such as caspase activation, chromatin condensation and DNA fragmentation,\(^5,6\) Δψ\textsubscript{m} loss is used as a marker to detect apoptosis.

Apoptosis is thought to contribute to the loss of pancreatic β-cells in the pathogenesis of type 1 diabetes.\(^6\) Key mediators of β-cell apoptosis are the cytokines interleukin-1β (IL-1β) and interferon-γ (IFN-γ) released by islet-infiltrating mononuclear cells in the ‘insulitis’ lesion. The pro-apoptotic action of these cytokines has been attributed to pleiotropic mechanisms mediated by a network of transcription factors and genes that impact glucose
metabolism, ATP balance, insulin biosynthesis and release. Furthermore, cytokine-mediated expression of the inducible form of nitric oxide synthase (iNOS) and the consequent synthesis of the radical nitric oxide (NO) contribute significantly to cytokine-induced β-cell dysfunction and apoptosis.

Mitochondria are of central importance for the function of insulin-producing β-cells and are considered to be substrate sensors that couple glucose metabolism to insulin secretion. In apoptosis of β-cells, mitochondria might be affected by cytokine-mediated activation of the intrinsic or extrinsic pathways, as well as by disturbances of ATP homeostasis. IL-1β and IFN-γ trigger apoptosis by the extrinsic pathway via induction of the death receptor Fas, and the intrinsic pathway via induction of iNOS, leading to generation of the β-cell-toxic radical NO. In the extrinsic pathway, cytokine-induced Fas receptor interacts with Fasl of activated T cells, leading to activation of pro-caspase 8 and subsequent activation of executioner caspases such as caspase-3. Alternatively, caspase 3 and 8 can be activated via an amplification loop involving mitochondria, which amplifies the caspase signal. Intriguingly, when RINm5F insulinoma cells were transfected with the anti-apoptotic factor Bcl-2 that prevents pro-apoptotic proteins, e.g. Bax, from inserting into mitochondrial membranes, Δψm impairment, caspase activation and cell death were inhibited.

The biochemical pathways by which β-cells undergo apoptosis are poorly defined. Our aim was to elucidate further the mechanisms by which cytokines activate apoptosis by the extrinsic and/or intrinsic pathways in β-cells by examining the effect of IL-1β and IFN-γ on Δψm in glucose-responsive NIT-1 cells and in the presence and absence of FasL.

Materials and methods

Reagents

All reagents were obtained from Sigma (Taufkirchen, Germany) unless otherwise specified. Super-Fasl, general caspase inhibitor (ZVAD), caspase 1 inhibitor (YVAD), caspase 3 inhibitor (DEVD), caspase 6 inhibitor (VEID) and caspase 8 inhibitor (IETD) were purchased from Alexis (San Diego, CA, US). Cytokines (recombinant murine IL-1β and IFN-γ) were obtained from TEBU (Frankfurt/M. Germany). Fetal calf serum and arginine-free RPMI-1640 along with FCS, glucose, glutamine, inositol, cysteine, leucine and methionine were from BioWhittaker (Verviers, Belgium). Fetal calf serum and arginine-free RPMI-1640 supplemented with FCS, glucose, inositol, cysteine, leucine and methionine. Cell-permeable and irreversible caspase inhibitors (ZVAD, YVAD, DEVD, VEID, IETD) were dissolved in dimethyl sulfoxide (DMSO) and added 30 minutes before cytokine treatment at 100 µM (final DMSO concentration during cell culture 0.2%). Cells were harvested by trypsinization.

Cytofluorometric determination of Δψm

Δψm was analysed by the method of Zamzami et al. Briefly, DiOC6 was dissolved in DMSO and stored as a stock solution (40 µM) at -20°C. DiOC6-working solution (400 nM in PBS) was freshly prepared for every experiment. CCCP, a protonophore known to disrupt Δψm, was used as a positive control at a final concentration of 100 nM (20 mM CCCP stock diluted in ethanol). After harvesting, cells were kept on ice until staining. Samples (5 x 10⁴ cells) were incubated with DiOC6 (20 nM) for 10 minutes at 37°C in a water bath. The tubes were immediately returned to ice and Δψm was analysed within 10 minutes by flow cytometry in the logarithmic green fluorescence channel (FL1). To determine the percent of NIT-1 cells with impaired Δψm, each staining assessment included a negative control without DiOC6 to check autofluorescence, an untreated sample with DiOC6 to set the region with unaffected Δψm, and a positive control with cells labelled in the presence of CCCP to define cells with impaired Δψm. Samples were analysed with an EPICS Profile II flow cytometer (Coulter, Krefeld, Germany) equipped with an argon laser at 15 mW. EPICS Profile Software II (version 3.0) was run for data acquisition as well as analysis.

Cell death

Cell death was determined as propidium iodide (PI) positivity. Cells were incubated with PI solution (50 µg/ml in phosphate-buffered saline (PBS)) and analysed on a Coulter EPICS flow cytometer.
Chromatin condensation
NIT-1 cells were fixed at 4°C in 4% phosphate-buffered paraformaldehyde for 30 minutes. After washing, cells were permeabilised using BD FACS Permeabilizing Solution 1 as recommended by the supplier, washed again, and stained with DAPI (50 ng/ml) at 37°C for 20 minutes. Analysis was performed using an Axioscope fluorescence microscope (Zeiss; Jena, Germany). Viable cells were identified by intact nuclei and apoptotic cells by fragmented nuclei. A minimum of 200 cells was counted in each experimental condition.

Flow cytometric analysis of Fas expression
Surface Fas expression on viable NIT-1 cells was analysed with the hamster anti-mouse Fas antibody Jo2 (2 µg/ml), with hamster immunoglobulins (2 µg/ml) as a negative control, as previously described.19 Jo2 immunoreactivity was detected by biotinylated anti-hamster IgG (Dianova, Hamburg, Germany) and Streptavidin-PE (BD Pharmingen, Heidelberg, Germany). The samples were analysed on an EPICS/XL flow cytometer.

Measurement of nitric oxide synthesis
NO production was measured by accumulation of nitrite in the culture medium, according to Zumsteg et al.20 In brief, 90 µl of cell-free medium were mixed with 10 µl of Griess reagent (one part 0.5% naphtylethylene diamine dihydrochloride and one part 5% sulfanilamide in 25% H3PO4) and incubated for 30 minutes at room temperature. Nitrite concentration was determined in triplicate within a concentration range that corresponded to the linear part of the standard curve. The intra-assay coefficient of variation (CV) was 6.2% and the inter-assay CV 6.1%. Absorbency was measured at 550 nm on an Elx800 microplate reader (Bio-TEK® Instruments, Inc., Vermont, US).

Measurement of inducible NO synthase (iNOS) mRNA
To measure the induction and variation of iNOS transcript levels in NIT-1 cells after various treatments, relative quantification by real-time RT-PCR was performed. Total cellular RNA was isolated from trypsinised NIT-1 cells with acid guanidinium isothiocyanate-phenol-chloroform (TRIzol®). To avoid contamination with genomic DNA, total RNA was treated with DNase (DNA-free™). Concentration and purity of total DNAse-treated RNAs were determined spectrophotometrically by measuring absorbance at 260 nm and the A260/280 ratio, respectively. To assess the integrity of prepared RNAs, samples were subjected to 1% agarose gel electrophoresis. The relative expression of the iNOS transcript was evaluated by RT-PCR using 18S ribosomal RNA (18S rRNA) as an internal standard. Two-step RT-PCR reactions were performed using the TaqMan reverse transcription reagent for the first strand cDNA synthesis. For each RNA sample, a 0.5 µg aliquot was reverse transcribed into cDNA with random hexamer primers and Multiscribe RT in a total volume of 100 µl. Real-time PCR amplification was performed with TaqMan Universal PCR Master Mix using 2 µl of cDNA as template and the 5700 Sequence Detection System. The forward primer (5'-GCT GGA AGC CAC TGA CAC TT-3') was used at a final concentration of 50 nM, and the TaqMan probe (5'-6FAM-CAC CTA CCCGACCCCGAGA x TGG-3' (X=TAMRA)) was used at 150 nM final concentration. Design of synthetic oligonucleotide primers and the fluorescent probe was based on published sequences (accession number M84373.1) and oligonucleotides were compared with public databases to check for specificity. The standard curve method with serially diluted cDNA from cytokine-treated NIT-1 cells was used for relative quantification of gene expression. RT-PCR experiments were performed in triplicate.

Statistical methods
Results were expressed as means ± SE. Statistical significance was assessed using the Student t-test. When multiple tests were performed, a Bonferroni-Holm correction was used to adjust the level of significance. Regression analysis was performed to identify slopes for concentration dependency. Differences were considered to be statistically significant at p<0.05.

Results
Cytokine-induced impairment of ΔΨm in NIT-1 cells
We first assessed the effects of cytokines on ΔΨm in NIT-1 cells exposed for up to 48 hours to a range of concentrations of IL-1β/IFN-γ. Both time- and concentration-dependent impairment of ΔΨm were observed. Significant impairment in ΔΨm compared to t0 was first observed after six hours and continued up to 48 hours (figure 1a). Impairment of ΔΨm was observed across a range of IL-1β/IFN-γ concentrations (10/100, 25/250, 50/500 and 100/1,000 U/ml) (figure 1b), starting at 10/100 U/ml (p<0.01). Regression analysis indicated a linear concentration effect between 10/100 U/ml and 100/1,000 U/ml of IL-1β/IFN-γ (p<0.01) (slope=0.147; r=0.983). In addition to impairment of ΔΨm, cytokine exposure (100/1,000 U/ml) of NIT-1 cells for 24 hours also induced surface Fas expression, cell death and nitrite accumulation (table 1).

Dominance of cytokine- over Fasl-induced ΔΨm impairment
Co-exposure to Fasl had no apparent effect on the time course of cytokine-induced ΔΨm impairment. After 24 hours, Fasl increased ΔΨm impairment in cytokine-treated NIT-1 cells and controls (figure 1). However, difference contrast analysis revealed that this Fasl effect on ΔΨm impairment was the same in untreated and cytokine-treated cells. Despite this, we found that Fasl had a significant additive effect on cell death of cytokine-treated cells (table 1).
We next investigated the impact of FasL on Δψ₁₄₃ in NIT-1 cells exposed to different concentrations of IL-1β/IFN-γ alone or in combination with FasL (figure 1b).

**ZVAD, VEID and IETD restore IL-1β/IFN-γ-induced impairment of Δψ₁₄₃**

The contribution of caspases to impairment of Δψ₁₄₃, nitrite accumulation and cell death in response to IL-1β/IFN-γ alone or in combination with FasL was assessed using the pan-caspase inhibitor ZVAD. The inhibitor alone had no effects on tested parameters (data not shown). Notably, cytokine-induced Δψ₁₄₃ impairment and cell death were reduced in the presence of ZVAD, and nitrite accumulation was enhanced by ZVAD (figure 2). Similarly, ZVAD reduced Δψ₁₄₃, impairment and cell death as well as increasing nitrite accumulation in the presence of FasL+IL-1β/IFN-γ.

To dissect further the role of caspases in cytokine-induced Δψ₁₄₃ impairment, the effects of the caspase 1 inhibitor YVAD, the caspase 3 inhibitor DEVD, the caspase 6 inhibitor VEID and the caspase 8 inhibitor IETD were also analysed. The inhibitors themselves had no effects on tested parameters (data not shown). Cytokine-induced Δψ₁₄₃ impairment was reduced in the presence of the caspase 6 inhibitor VEID and the caspase 8 inhibitor IETD (figure 2). Δψ₁₄₃ impairment induced by cytokines+FasL was reduced by treatment with YVAD and IETD.

**Effect of iNOS inhibition on Δψ₁₄₃ in cytokine-induced apoptosis of NIT-1 cells**

We next assessed the effect of inhibiting iNOS on cytokine-
induced apoptosis. NIT-1 cells were exposed to 100/1,000 U/ml of IL-1β/IFN-γ for 24 hours and treated with the iNOS inhibitor L-NAME. Compared to nitrite accumulation in samples treated with cytokines alone (1.5±0.4; n=5), levels were reduced upon co-treatment with L-NAME (0.18±0.04; n=5) to those of controls (0.15±0.03; n=5), indicating that L-NAME prevented cytokine-induced nitrite accumulation. In addition, cytokine-induced Δψm impairment (81.6±7.7; n=3) was also reduced in the presence of L-NAME (52.4±8.6; n=3; p<0.01), similar to the level of untreated controls (45.1±5.8; n=4).

Nitrite accumulation in cytokine-induced and FasL-induced apoptosis of NIT-1 cells

IL-1β/IFN-γ induced a time- and concentration-dependent accumulation of nitrite in the culture medium (figure 1). When cells were exposed to 100/1,000 U/ml of IL-1β/IFN-γ for 48 hours (figure 1a), significant nitrite accumulation was observed after six hours. Concentration dependency was tested after 24 hours’ exposure, when 25/250 U/ml IL-1β/IFN-γ was observed to induce a significant increase in nitrite levels (p<0.01; figure 1b). Consistent with measured nitrite accumulation (figure 1; table 1), real-time RT-PCR analysis clearly confirmed a specific induction of iNOS mRNA after treatment with 100/1,000 U/ml IL-1β/IFN-γ in NIT-1 cells (data not shown). A time- and concentration-dependent accumulation of nitrite was also observed in the presence of FasL+IL-1β/IFN-γ (figure 1).

To investigate the potential additional effect of FasL on NO generation, we measured iNOS mRNA and nitrite levels in NIT-1 cells exposed to 100/1,000 U/ml IL-1β/IFN-γ+FasL. At this concentration of cytokines, we did not detect a significant effect of FasL on nitrite generation as assessed by iNOS or nitrite levels. However, at 25/250 U/ml IL-1β/IFN-γ, FasL significantly increased the accumulation of nitrite (figure 1b).

Discussion

Using the glucose-responsive NIT-1 insulinoma cell line as a model for pancreatic β-cells, we sought to understand better the mechanism of cytokine-induced β-cell apoptosis by investigating the downstream effects of cytokines alone or in combination with FasL on mitochondrial transmembrane potential and induction of apoptosis. The cytokine-induced impairment of Δψm in NIT-1 cells accords with reports of the effects of IL-1β, IFN-γ and TNF-α in the insulinoma cell lines INS-1 and RINm5F. We have also confirmed this effect in BB/OK rat islets exposed to IL-1β, IFN-γ and TNF-α (P Augstein, unpublished results), as reported previously for human and rat islets exposed to IL-1β2,24 or the combination of IL-1β, IFN-γ and TNF-α.25 The cytokine-induced impairment is therefore a general feature of cytokine-induced β-cell death.

In contrast to cytokine-induced impairment of Δψm, the effect of FasL on Δψm has not been previously reported. Similar to our earlier reports, addition of FasL to cytokine-exposed NIT-1 cells enhanced cell death, implicating a cytokine-induced Fas and FasL interaction and intact downstream Fas signalling pathways. However, FasL alone had only a minor effect on Δψm. Comparison of Δψm impairment induced by different concentrations of IL-1β/IFN-γ alone or with the addition of FasL revealed a clear dominance of cytokine-induced effects. This suggests that the extrinsic apoptotic pathway mediated by FasL has only a minor impact on Δψm in NIT-1 cells. One interpretation...
relates to the nature of clonal β-cells. \( \Delta \psi_m \) is affected very differently in other models of FasL-induced apoptosis, depending on the cell line and induction agent.\(^6\) In our model, in which cytokines and Fasl act simultaneously, the dominant cytokine effect might precede and override any effect by Fasl. Another possibility is that β-cell mitochondria are unresponsive to FasL-induced effects. Koskin et al.\(^{27}\) reported that β-cells have a mitochondrial permeability transition which differs remarkably at least in clonal β-cells.

As is well known\(^{10}\) and confirmed here, IL-1β and IFN-γ induce NO, which is thought to act on mitochondria by several mechanisms and to function as a second messenger for apoptosis.\(^5\) Treatment with L-NAME, an inhibitor of iNOS, resulted in a reduction of \( \Delta \psi_m \) impairment, suggesting that NO plays a major role in mediating \( \Delta \psi_m \) impairment associated with cytokine-induced apoptosis of NIT-1 cells. Similarly, L-NAME prevented disruption of \( \Delta \psi_m \) in RINm5F cells.\(^6\) In rat islets, NO has been found to inhibit mitochondrial enzymes and to suppress mitochondrial activity.\(^8\) Remarkably, analysis of the gene network regulating cytokine-induced apoptosis has revealed that a major group of genes affected by NO are related to metabolism and ATP production.\(^7\) Indeed, the impact of NO on \( \Delta \psi_m \) in β-cells had been shown in studies using alloxan, the NO donor SNAP or sodium nitroprusside in INS-1 cells,\(^{11}\) RINm5F cells\(^5\) and rat islets.\(^5,11\) Future studies could employ the iNOS\(^{2,3}\) knock-out mouse\(^5\) which, despite the absence of NO generation, exhibits cytokine-induced apoptosis.

Intriguing, the general caspase inhibitor ZVAD augmented nitrite generation but reduced \( \Delta \psi_m \) impairment and cell death. This dichotomy indicates unexplained mechanisms. Indeed, depending on the cell line investigated, caspase inhibition by ZVAD influences signalling by transcription factor NF-κB, c-Jun N-terminal protein kinase (JNK), mitogen-activated protein kinase phosphatase-1 (MKP-1) or extracellular signal-regulated kinases (ERKs).\(^{12,15}\) Interaction(s) between these pathways need(s) to be revealed in future studies.

Fasl modulated the generation of NO depending on cytokine concentration. At 25/250 and 50/500 U/ml cytokines, Fasl had an additive effect on NO accumulation. However, at 100/1,000 U/ml, the cytokine-mediated effect was dominant and NO generation was not affected by Fasl, as revealed by medium nitrite accumulation and iNOS mRNA. NO is known to mediate its effects via activation of the transcription factor nuclear factor-κB.\(^{14}\) Our findings suggest there is cross-talk between the Fasl-triggered mechanisms and the induction of iNOS by sub-optimal concentrations of cytokines.

To address the importance of caspases for cytokine-induced \( \Delta \psi_m \) impairment, we performed experiments in the presence of inhibitors of caspases 1, 3, 6 and 8. ZVAD stabilised \( \Delta \psi_m \) in the presence of cytokines, implicating a central role for caspases in cytokine-induced apoptosis of β-cells. In the general model, the initiator caspase 8 activates the caspase cascade in response to the Fasl/Fas interaction.\(^1\) Consistent with this view, the caspase 8 inhibitor IETD reduced cell death in the presence of Fasl and cytokines. Moreover, our experimental dissection of caspase function revealed that inhibition of caspase 8 by IETD prevented cytokine-induced \( \Delta \psi_m \) impairment. Notably, cytokine-induced apoptosis of βTC1-Tet cells, a highly differentiated insulin-secreting cell line, was associated with activation of caspase 8,\(^{15}\) suggesting that caspase 8 contributes to cytokine-induced apoptosis of β-cells. Recent findings in KB cells that demonstrate cross-talk between Fas and IFN-γ signalling provide an explanation for our findings,\(^{16}\) in that IFN-γ sensitises for apoptosis by facilitating caspase 8 processing by death-inducing signalling complex (DISC).\(^{17}\) Another explanation would be the activation of caspase 8 by a post-mitochondrial amplification loop,\(^{18}\) which relies on a proteolytic cleavage promoted by the effector caspases.

Caspase 1 was the first identified caspase, and is the prototype inflammatory caspase.\(^{19,20}\) Although caspase 1 is not required for apoptosis and diabetes development,\(^{4,21}\) it is involved in β-cell damage and enhancement of NO-dependent pathways.\(^{22,23}\) The caspase 1 inhibitor YVAD reduced cytokine-induced \( \Delta \psi_m \) impairment in the presence of FasL. Caspase 1 could affect \( \Delta \psi_m \) by at least two distinct pathways: by activation of NF-kappaB and p38 MAPK\(^{24}\) or by recruitment to the inflammasome.\(^{25}\) Nevertheless, little is known about the importance of caspase 1 in the intrinsic pathway in β-cell apoptosis and its function in this process warrants further investigation.

In addition to the effect on \( \Delta \psi_m \), IL-1β combined with IFN-γ\(^{26}\) or TNF-α\(^{27}\) has been found to activate caspase 3 in NIT-1 cells\(^{28}\) and RINm5F cells.\(^5\) In contrast to studies in RINm5F and Min6 cells,\(^5\) the DEVD inhibitor of caspase 3-like proteases did not affect NIL-1 cell death. Similarly, treatment with DEVD could not restore viability of HIT-15 cells treated with mycophenolic acid for GTP depletion.\(^{29}\) Whether activated caspase-3 has a pro-apoptotic or an alternate prosurvival role needs to be researched in future studies. DEVD also did not reduce cytokine-induced \( \Delta \psi_m \) impairment. Caspase 6 has a variety of substrates including caspase 3, lamins, keratin-18 and PARP.\(^{30}\) The functional contribution of each caspase in apoptotic pathways appears to vary among the different models of apoptosis.\(^{31}\) Moreover, inhibition of some signal-transduction events required for DNA degradation in apoptosis does not prevent cell death.\(^{32}\) Taken together, our findings suggest that caspases 1, 6 and 8 function to promote \( \Delta \psi_m \) impairment in cytokine-induced apoptosis. However, the intrinsic apoptotic pathway remains poorly defined for β-cells.

The main conclusions of our study are: (1) the cytokines IL-1β/IFN-γ strongly affect mitochondria and prime clonal NIT-1 β-cells for the intrinsic apoptotic pathway; (2) NO and caspase activation contribute to cytokine-induced \( \Delta \psi_m \) impairment; and (3) Fasl combined with IL-1β/IFN-γ augments cytokine-induced cell death by mechanisms that do not involve impairment of \( \Delta \psi_m \).

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Conflict of interest statement
None declared.

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