Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis

Inna N. Lavrik, Alexander Golks, Simone Baumann, and Peter H. Krammer

Introduction

CD95 (APO-1/Fas) is a member of the death receptor family, a subfamily of the TNF-R superfamily.\(^1\)\(^,\)\(^,\)\(^2\) Crosslinking of CD95 with its natural ligand CD95L (FasL/CD178)\(^6\) or with agonistic antibodies such as anti–APO-1\(^7\) induces apoptosis in sensitive cells. The death-inducing signaling complex (DISC) is formed within seconds after CD95 stimulation.\(^8\) The DISC consists of oligomerized, probably trimerized, CD95 receptors; the adaptor molecule FADD; 2 isoforms of procaspase-8 (procaspase-8a and procaspase-8b); procaspase-10; and c-FLIP\(^L,\)\(^5\)\(^,\)\(^9\). The interactions between molecules at the DISC are based on homotypic contacts. The death domain (DD) of the receptor interacts with the DD of FADD, whereas the death effector domain (DED) of FADD interacts with the N-terminal tandem DEDs of procaspase-8, -10, and c-FLIP\(^L,\)\(^5\)\(^,\)\(^9\). The binding of procaspase-8 to the DISC results in processing of the zymogen. As a result the active caspase-8 heterotetramer \(\text{p102-p182}\) is released into the cytosol to propagate the apoptotic signal.\(^10\)

Two CD95 signaling pathways were established.\(^1\) Type I cells are characterized by high levels of CD95 DISC formation and increased amounts of active caspase-8. Activated caspase-8 directly leads to the activation of downstream effector caspase-3 and -7. Type II cells are characterized by lower levels of CD95 DISC formation and, thus, lower levels of active caspase-8.\(^1,\)\(^11\) In this case, signaling requires an additional amplification loop that involves the cleavage by caspase-8 of the Bcl-2-family protein Bid to generate truncated (t) Bid and subsequent tBid-mediated release of Cytochrome C from mitochondria. The release of Cytochrome C from mitochondria results in apoptosome formation, followed by activation of procaspase-9, which in turn cleaves downstream, effector caspases.\(^12\)

Caspase-2 is referred to as an initiator caspase.\(^1,\)\(^3\)-\(^15\) It is characterized by the presence of a caspase activation recruitment domain (CARD) and is structurally related to the initiator caspase-9.\(^16\)\(^,\)\(^17\) The mechanism of procaspase-2 activation in apoptosis in contrast to other initiator caspases remains poorly defined. It was reported that caspase-2 is implicated in Cytochrome C release and is essential for drug-induced apoptosis in several human cell lines.\(^18\)-\(^20\) On the basis of these data caspase-2 was proposed to be the initiator caspase in cytotoxic stress-induced apoptosis acting upstream of mitochondria. At the same time, others suggested that activation of caspase-2 occurs downstream of mitochondria.\(^21\) In addition, caspase-2 was reported to be a proximal mediator in the heat-shock-induced apoptosis.\(^22\)

The role of caspase-2 in death receptor signaling is also contradictory. Caspase-2 was shown to be processed in the course of death receptor–mediated apoptosis.\(^23\),\(^24\) It was reported that this cleavage is mediated by caspase-3 and, accordingly, processing of caspase-2 takes place after activation of caspase-3. In contradiction to these data, caspase-2 was shown to be necessary for optimal TRAIL-mediated cleavage of Bid.\(^25\) Down-regulation of caspase-2 using RNA interference sufficiently inhibited TRAIL-induced apoptosis in type II cells. Moreover, it was demonstrated that in caspase-2–knockdown cell lines the sensitivity toward CD95-mediated apoptosis is decreased.\(^26\) In the latter work, it was suggested that caspase-2 influences activation of procaspase-8 at the DISC. However, the investigators failed to detect an association of caspase-2 with the DISC or with FADD and procaspase-8.

To clarify the contradictions in the literature, we analyzed the role of procaspase-2 in CD95-mediated apoptosis. We show that procaspase-2 is found at the CD95 DISC and is activated on CD95 stimulation in a number of human T- and B-cell lines. At the same time we demonstrate that procaspase-2 in the absence of procaspase-8 could not initiate cell death. Thus, we have shown that caspase-2 is activated at the DISC but does not prime CD95-induced apoptosis.
Materials and methods

Cell lines

The T-cell lines HUT78, CEM, Jurkat (caspase-8 deficient, clone I9.2), Jurkat clone A3, and the B-lymphoblastoid cell lines SKW6.4, Raji, BJAB were maintained in RPMI 1640 (Life Technologies, Germany), 10 mM HEPES (Life Technologies, Germany), 50 μg/mL gentamicin (Life Technologies, Karlsruhe, Germany), 10% fetal calf serum (Life Technologies) in 5% CO2. The caspase-8 deficient Jurkat cell line (clone I9.2) and Jurkat clone A3 were obtained from J. Blenis (Harvard Medical School).

Antibodies and reagents

Anti–caspase-2 monoclonal antibody (clone 35; mouse IgG1), anti-FADD monoclonal antibody (mouse IgG1) were purchased from Transduction Laboratories (Lexington, KY). Anti–CD95, anti-RAIDD polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD95, anti-RAIDD polyclonal antibody was from Biosource International (Nivelles, Belgium). The anti–caspase-8 mAb C15 (mouse IgG2b) recognizes the p18 subunit of caspase-8. Anti–APO-1 is an agonistic monoclonal antibody (IgG3, κ) recognizing an epitope on the extracellular part of CD95 (APO-1/Fas). The horseradish peroxidase–conjugated goat anti–mouse IgG1, IgG2a, and IgG2b were from Southern Biotechnology Associates (Manchester, United Kingdom). Leucine zipper (LZ)–CD95L was produced as described.27 ZVAD-fmk was purchased from Merck (Darmstadt, Germany). ZIETD-afc and zVDVAD-afc were from Molecular Probes (Leiden, The Netherlands). Caspase-2 siRNA and caspase-8 siRNA (SI00038542 and SI00299593, respectively) were purchased from Qiagen (Hilden, Germany). HiPerFect Transfection Reagent was purchased from Qiagen. All other chemicals used were of analytical grade and purchased from Merck or Sigma (Taufkirchen, Germany).

Flow cytometry analysis

The percentage of viable cells was determined by forward light scatter/ sideward light scatter (FSC/SSC) using a fluorescence-activated cell scan (FACScan) Cytometer (BD, Heidelberg, Germany). A minimum of 10 000 cells per sample was analyzed. Specific cell death was calculated as follows: (percentage of experimental cell death – percentage of spontaneous cell death) / (100 – percentage of spontaneous cell death) × 100.

Immunoblotting

For immunoblotting analysis the cells were lysed in buffer A (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [Sigma], protease inhibitor cocktail [Roche, Basel, Switzerland]) for 15 minutes on ice and centrifuged [15 minutes at 14 000g]). Postnuclear supernatant equivalents of 0.5 × 106 cells or 25 μg protein as determined by the bicinechonic acid (BCA) method (Pierce, Rockford, IL) were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane (Amersham, Uppsala, Sweden).

Caspase activity assays

Cytosolic lysates were incubated with 50 μM site-specific tetrapeptide substrates (zIETD-afc for caspase-8, zVDVAD-afc for caspase-2) in a caspase assay buffer B (50 mM HEPES, 100 mM NaCl, 10 mM dithiothreitol, 0.1% [wt/vol] CHAPS, 10% [wt/vol] sucrose, pH 7.4) in a final volume of 200 μL. The release of the fluorogenic group AFC was determined after 1 hour of incubation at 37°C by a microplate fluorescence reader Wallach 1420 (Perkin Elmer, Langen, Germany) at the excitation wavelength of 405 nm and emission wavelength of 535 nm.

DISC analysis by immunoprecipitation and immunoblotting

The composition of CD95 DISC was determined as follows: 1 × 106 cells were either treated with 10 μg/LZ-CD95L–containing supernatant or 1 μg/mL anti–APO-1 (IgG3) for 5 minutes (unless otherwise indicated) at 37°C, washed twice in 1 × PBS, and subsequently lysed in buffer A (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail [Roche], 1% Triton X-100 [Serva, Heidelberg, Germany], and 10% glycerol) (stimulated condition) or lysed without treatment (unstimulated condition). CD95 DISC was immunoprecipitated overnight with 2 μg anti–APO-1 and 30 μg Protein-A Sepharose. Beads were washed 5 times with 20 volumes of lysis buffer. The immunoprecipitates were analyzed on 12% PAAG. Subsequently, the gels were transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany), blocked with 5% nonfat dry milk in PBS/Tween (PBS plus 0.05% Tween 20) for 1 hour, washed with PBS/Tween, and incubated with the primary antibody in PBS/Tween at 4°C overnight. Blots were developed with a chemiluminescence method following the manufacturer’s protocol (PerkinElmer Life Sciences, Rodgau, Germany).

Caspase-2 immunoprecipitation

The Caspase-2 immunoprecipitation was performed as described for DISC immunoprecipitation. Briefly, 1 × 106 cells were treated with 10 μg LZ-CD95L–containing supernatant for 10 minutes at 37°C, washed twice in 1 × PBS, and subsequently lysed in buffer A (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail [Roche], 1% Triton X-100 [Serva], and 10% glycerol). Caspase-2 was immunoprecipitated overnight with 5 μg anti-caspase-2 antibodies and 30 μL Protein-A Sepharose.

Caspase inhibition experiments

ZVAD-fmk (pan-specific caspase inhibitor) was added to the cells 30 minutes before the addition of anti–APO-1 or LZ-CD95L.

siRNA experiments

Transfections of siRNA were performed according to the manufacturer’s protocol using HiPerFect Transfection Reagent (Qiagen).

Results

Caspase-2 is processed during CD95-mediated apoptosis

Caspase-2 was reported to be processed in the course of CD95-mediated apoptosis.23,24 To further investigate these findings, B-lymphoblastoid SKW6.4 cells were stimulated with 5 μg/mL agonistic anti-CD95 (anti–APO-1) antibody for various time points. CD95-mediated processing of caspase-2 was analyzed by Western blotting using anti-caspase-2 mAbs (Figure 1A-B). Cleavage of caspase-2 to the p33 subunit was already observed 5 minutes after stimulation similar to caspase-8 processing (Figure 1A). Furthermore, maximal cleavage of both caspases appeared at 20 minutes after triggering of CD95. To analyze caspase processing at a slower kinetics we stimulated SKW6.4 cells with 200 ng/mL anti–APO-1 (Figure 1C). In this case, a significant increase in caspase processing could only be detected after 2 hours. In summary, we observed that the cleavage kinetics of caspase-2 and -8 were identical on strong and weak CD95 stimulation.

To confirm this observation in another cell line we analyzed the CD95-mediated processing of caspase-2 and -8 in CEM cells. CEM cells are referred to as type II cells that are characterized by lower levels of DISC formation and, subsequently, by different kinetics of cell death. Indeed, we observed a slower activation of caspases on stimulation with 5 μg/mL anti–APO-1 (Figure 1D). The first cleavage products of both caspases appeared only after 1 hour. Again the processing of both caspases took place simultaneously. We also observed parallel activation of caspase-8 and -2 in other
cell lines: in H9 (type I cells) (Figure 1E), HUT78 (type I cells) (data not shown), and in Jurkat A3 (type II cells) (data not shown).

To verify that procaspase-2 is not only cleaved in the course of CD95-mediated apoptosis but also undergoes proteolytic activation we implemented fluorometric caspase activity assays with a fluorescent substrate of caspase-2, zVDV AD-afc. After treatment of SKW6.4 cells with different concentrations of anti–APO-1 antibody we observed an increase in fluorescence on activation that clearly indicated activation of caspase-2 (Figure 1F). Activation of caspase-2 was paralleled by activation of caspase-8 measured using zIETD-afc as a substrate (Figure 1F). Catalytic activation of both caspases, caspase-2 and -8, was inhibited by addition of the pancaspase inhibitor zV AD-fmk (Figure 1G).

To ensure that simultaneous activation of both caspases takes place also with a different trigger of CD95 we stimulated SKW6.4 cells with LZ-CD95L (Figure 1H). Again, we observed simultaneous appearance of caspase-2 and -8 cleavage products, and their processing was inhibited by zVAD-fmk.

Taken together, we demonstrate that kinetics of caspase-2 and caspase-8 activation is identical and that caspase-2 activation occurs at the early stages of CD95-mediated apoptosis.

Caspase-2 is recruited to the DISC

To determine at which step in CD95 signaling activation of procaspase-2 takes place we analyzed CD95 DISC formation. HUT78 cells were stimulated with agonistic anti–APO-1 antibody. The CD95 DISC was immunoprecipitated with anti–APO-1 antibody and analyzed by immunoblotting. In contrast to previous reports, procaspase-2 was detected at the CD95 DISC (Figure 2A). We probed the immunoprecipitated complex for the presence of other CD95 DISC components. Immunoblotting analysis revealed the presence of CD95 and procaspase-8/a and -8/b along with their cleavage products p43/p41 (Figure 2A).

To analyze whether procaspase-2 is catalytically activated at the DISC we tested an in vitro–isolated DISC for specific caspase-2 catalytic activity. CD95 receptor was immunoprecipitated with anti–APO-1 antibody and Protein-A beads both from CD95-stimulated as well as from unstimulated SKW6.4 cells. Subsequently, the beads, containing the CD95 DISC, were incubated with zVDVAD-afc, a fluorescent substrate of caspase-2 (Figure 2B). We detected an increase in fluorescence on stimulation, which
Procaspase-2 is present at the DISC of several cell lines

Following this finding, we analyzed CD95 DISCs, immunoprecipitated from different B- and T-cell lines. We could detect caspase-2 in CD95 DISCs of various cell lines (Figure 3A). Caspase-2 was detected in the CD95 DISC of type I cells (SKW6.4, HUT78, Raji, BJAB) as well as in the DISC of type II cells (CEM, Jurkat A3). Not only the full-length form of procaspase-2 was detected at the DISC, but also the p33 subunit that results from the first cleavage step of procaspase-2 (Figure 1B). Interestingly, the amount of caspase-2, detected at the DISC in HUT78 cells, was lower compared with the one in SKW6.4 cells despite similar recruitment of procaspase-8. However, we observed a slightly enhanced recruitment of procaspase-2 to the DISCs from Jurkat A3 and CEM cells referred to as type II cells.

The specificity of CD95 DISC formation was controlled, probing for other components of the DISC with specific antibodies against CD95, FADD, and procaspase-8. Immunoblotting revealed the presence of CD95 as well as FADD, and products of procaspase-8/a and -8/b processing (p55/p53, p43/p41, and p18). Notably, the amount of procaspase-2 that was recruited to the DISC from the lysates was always lower than the corresponding amounts of FADD and procaspase-8 (Figure 3A; data not shown). Interestingly, in unstimulated cells we could not detect an association of procaspase-2 with individual DISC components, c-FLIP, and procaspase-8, when performing immunoprecipitations using anti-FLIP mAb NF6 and anti–caspase-8 mAb N1 (data not shown).
To further verify that caspase-2 is a component of the CD95 DISC complex in several human T- and B-cell lines we stimulated SKW6.4, HUT78, BJAB, CEM, and Jurkat A3 cells with LZ-CD95L, which was followed by immunoprecipitation using an anti–caspase-2 antibody. Indeed, we could communoprecipitate components of the CD95 DISC: CD95 and procaspase-8 (Figure 3B).

RAIDD has been reported to interact with caspase-2 by homotypic contacts of the respective CARD domains.29,30 Because RAIDD also possesses a DD it seems likely to be the adaptor molecule for procaspase-2 at the DISC. To check this hypothesis we analyzed CD95 DISCs that were immunoprecipitated from SKW6.4, HUT78, CEM cells for the presence of RAIDD. However, RAIDD was not observed at the DISC despite its presence in total cell lysates (data not shown). Thus, we demonstrate that procaspase-2 is found at the CD95 DISC of several human T- and B-cell lines, and we did not find RAIDD as an adaptor of caspase-2 at the DISC.

Procaspase-2 does not initiate apoptosis in the absence of caspase-8

Initiator caspases undergo autocatalytic activation when they are brought into close proximity. Following activation, initiator caspases start the apoptotic caspase cascade. To address the question whether procaspase-2 can initiate CD95-mediated apoptosis in the absence of caspase-8, we examined the caspase-8–deficient cell line Jurkat A3 (Figure 4A). Consistent with published data the treatment of the caspase-8–deficient cells with LZ-CD95L did not result in apoptosis up to 24 hours (Figure 4A).24 As control the cells from the parental Jurkat cell line A3 were treated with the same amount of LZ-CD95L, which efficiently caused apoptosis (Figure 4A). Similarly, no apoptosis was observed in caspase-8–deficient cells on stimulation with anti–APO-1 (Figure 4B). SKW6.4 cells that were used as a positive control exhibit high sensitivity on stimulation with the same amount of anti–APO-1 antibody.

Following this assay, we characterized the CD95-mediated status of procaspase-2 in caspase-8–deficient cells. Procaspase-2 was present in caspase-8–deficient cells according to the immunoblotting analysis (Figure 4C). However, we did not monitor procaspase-2 processing on stimulation with LZ-CD95L up to 2 hours (Figure 4C). In the parental Jurkat A3 cells, both procaspase-8 and -2 were cleaved on addition of LZ-CD95L (Figure 4C).

Furthermore, immunoprecipitation experiments that were done in parallel from the same total cellular lysates stimulated with LZ-CD95L have shown that procaspase-2 is recruited to the CD95 DISC of caspase-8–deficient cells (Figure 4D). Procaspase-8 was indeed absent in this cell line, whereas it was recruited and processed at the DISC in the parental cell line (Figure 4D).

**Figure 4. Procaspase-2 does not initiate apoptosis in the absence of procaspase-8.** (A) Caspase-8–deficient Jurkat A3 cells and parental Jurkat A3 cells were stimulated with LZ-CD95L for indicated periods of time. Cell death was measured by FACS using FSC/SSC. (B) Caspase-6–deficient Jurkat A3 cells and SKW6.4 cells were stimulated with indicated concentrations of anti–APO-1 for 24 hours. Cell death was measured by FSC/SSC. (A-B) Error bars indicate SD. (C) Caspase-8–deficient Jurkat A3 cells and parental Jurkat A3 cells were stimulated with LZ-CD95L for indicated periods of time. The total cellular lysates were analyzed by immunoblotting using anti–caspase-2 mAbs (anti-C2) and anti–caspase-8 mAbs (anti-C8). Unspecific bands, recognized by the anti–caspase-8 antibody in caspase-8–deficient Jurkat cells, are marked by stars. (D) Caspase-8–deficient and parental Jurkat A3 cells were stimulated with LZ-CD95L for indicated periods of time. CD95 was immunoprecipitated from 5 × 10⁶ cells using anti–APO-1 antibody with Protein-A Sepharose. Immunoprecipitates were subjected to 12% PAAG gels and immunoblotted with anti–caspase-2 mAbs and anti–caspase-8 mAbs. (E) Caspase-8–deficient Jurkat A3 cells were stimulated with LZ-CD95L for 1, 3, 4, and 6 hours. CD95 DISCs were immunoprecipitated from 3-, 4-, and 6-hour time points. Immunoprecipitates along with total cellular lysates were analyzed by immunoblotting using anti–caspase-2 mAbs and anti–FADD mAbs. (F) SKW6.4 and CEM cells were silenced using caspase-2 siRNA. Processing of procaspase-8 in these cells was analyzed after stimulation with 1 μg/mL anti–APO-1 for 1 hour. (G) SKW6.4 and CEM cells were silenced using caspase-8 siRNA. Processing of procaspase-2 as well as PARP cleavage were analyzed after stimulation with 1 μg/mL anti–APO-1 for 1 hour.
Importantly, we did not observe processing of procaspase-2 at the DISC, neither on short (Figure 4D) nor on longer stimulation times up to 6 hours (Figure 4E). Thus, we have found procaspase-2 at the CD95 DISC of caspase-8–deficient cells. Caspase-8–deficient Jurkat A3 cells were resistant toward CD95-mediated apoptosis, and the resistance was accompanied by the absence of procaspase-2 processing. This finding indicates that procaspase-2 is not an initiator caspase in CD95-induced apoptosis.

To rule out that this effect is cell-type specific we used siRNA for caspase-8 and caspase-2 in several type I and type II cell lines. Caspase-2 silencing did not influence caspase-8 activation in either type I or type II cell lines (Figure 4F). Interestingly, caspase-2 was not activated on CD95-stimulation in caspase-8–/– cell lines (Figure 4G). Not only was caspase-2 not activated in caspase-8–/– cell lines, but PARP cleavage also did not take place (Figure 4G) nor did caspase-3 and Bid cleavage (data not shown), indicating defects in CD95-induced apoptosis. Taken together, these data show that caspase-8 is the initiator caspase in the CD95-induced apoptosis and not caspase-2.

**Discussion**

The role of procaspase-2 in apoptosis is contradictory. Structurally it belongs to the family of initiator caspases that undergo activation when being brought into close proximity and, subsequently, start the apoptotic process. However, the absence of a severe phenotype in caspase-2–deficient mice has complicated the assignment of its role in apoptosis. Nevertheless, in several studies procaspase-2 has been reported to function as the initiator caspase in apoptosis induced by genotoxic stress. Recently, there have been reports on caspase-2 in death receptor-mediated apoptosis, which indicate that its function is more intriguing and beyond the function of one of the downstream caspases. It has been demonstrated that procaspase-2 must be activated shortly after receptor stimulation upstream of Bid cleavage and that down-regulation of caspase-2 results in a decrease of sensitivity toward death receptor-mediated apoptosis. In this study, we address the question of the mechanism of procaspase-2 activation in CD95-mediated apoptosis. We have found the activating complex for procaspase-2, and we show that this complex is indeed the CD95 DISC. Moreover, we have demonstrated that procaspase-2 contributes to the amplification of the death signal through Bid and the mitochondrial pathway.

Notably, other groups did not detect procaspase-2 in CD95 DISC immunoprecipitations under native conditions, using different antibodies and different time points for the analysis of the formation of the CD95 DISC. As procaspase-2 is recruited to the CD95 DISC in lower amounts than procaspase-8 and FADD (Figure 3A), it seems likely that more sensitive immunoprecipitation methods are required using anti–APO-1 antibodies.

Interactions between the molecules at the DISC are based on the contacts between homotypic domains. FADD is recruited to the DISC by DD interactions, and procaspase-8 and -10 are recruited to the DISC by DED interactions. Procaspase-2 possesses a CARD domain that is well described as an important apoptotic domain that comprises 6 antiparallel α-helices in different orientation as compared with DD and DED. The question arises what the putative adaptor molecule could be that can dock procaspase-2 to CD95 DISC. A likely candidate for the adaptor molecule is RAIDD, one of the few molecules that contains both a DD and a CARD. Furthermore, RAIDD has been reported to interact with procaspase-2 in the recently reported PIDDosome.
cells in which the concentration of active caspase-8 is not high enough to trigger apoptosis. Then participation of caspase-2 would be indispensable.

Other large protein complexes, which involve procaspase-2, have been reported. Procaspase-2 on overexpression has been shown to associate with RAIDD in the TNFR1 complex.\(^{30,40}\) However, in a recent analysis of the endogenous TNFR1 signaling complex neither procaspase-2 nor RAIDD have been found.\(^{28}\) Recently, an interesting cytosolic complex of caspase-2 has been described that comprises the adaptor molecule RAIDD and the p53-induced death domain-containing protein (PIDD).\(^{29}\) This complex has been suggested to function under genotoxic stress. In addition, procaspase-2 was shown to be recruited to the TRAF1, TRAF2, and RIP-containing protein complex that activates NF-κB through the CARD domain of caspase-2.\(^{41}\) Moreover, there is increasing evidence that triggering of CD95 does not only result in death signals but also in NF-κB activation.\(^{42}\) It is possible that procaspase-2 is a missing link between the CD95 DISC and CD95-mediated NF-κB activation. This question should be further addressed in future studies.

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### References

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