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A Role for the NF-κB Pathway in Cell Protection from Complement-Dependent Cytotoxicity

Dana Gancz, Michal Lusthaus, and Zvi Fishelson

Nucleated cells are equipped with several mechanisms that support their resistance to complement-dependent cytotoxicity (CDC). The role of the NF-κB pathway in cell protection from CDC was examined. Elevated sensitivity to CDC was demonstrated in cells lacking the p65 subunit of NF-κB or the IκB kinases IκKα or IκKβ, and in cells treated with p65 small interfering RNA. Pretreatment with the IκK inhibitor PS-1145 also enhanced CDC of wild-type cells (WT) but not of p65−/− cells. Furthermore, reconstitution of p65 into p65−/− cells and overexpression of p65 in WT cells lowered their sensitivity to CDC. The postulated effect of p65 on the JNK-mediated death-signaling pathway activated by complement was examined. p65 small interfering RNA enhanced CDC in WT cells but not in cells lacking JNK. JNK phosphorylation induced by complement was more pronounced in p65−/− cells than in WT cells. The results indicate that the NF-κB pathway mediates cell resistance to CDC, possibly by suppressing JNK-dependent programmed necrotic cell death.


Materials and Methods

Cells

Wild-type (WT), p65−/−, and IκKα−/− mouse embryonic fibroblasts (MEFs) prepared from WT, p65− (31), and IκKα− (32) knockout mice, respectively, were provided by Dr. Alexander Hoffmann (University of California, San Diego, La Jolla, CA), WT MEFs, IκKα knockout MEFs, IκKβ knockout MEFs, and IκKα- and IκKβ- double knockout MEFs (33, 34) were provided by Dr. Inder Verma (Salk Institute, San Diego, CA). HeLa and HEK 293T cells (American Type Culture Collection, Manassas, VA) and MEFs were maintained in DMEM supplemented with 10% heat-inactivated FBS (Life Technologies Laboratories, Grand Island, NY), 1% glutamine, 2% pyruvate, and an antibiotics mixture (Bio-Lab, Jerusalem, Israel) at 37°C and 5% CO2.

Sera, antisera, and reagents

Normal human serum (NHS) was prepared from healthy individuals and immediately frozen at −70°C until used. CS-deficient human serum (CSd-HS) was prepared from a CS-deficient patient. The serum served as a source for human complement and was not subjected to any analysis (hence an ethics approval was not required). Heat-inactivated NHS (HIS) was prepared by heating NHS at 56°C for 45 min. A polyclonal antiserum...
directed to K562 cells was prepared by immunization of rabbits and was heat-inactivated as above. This antiserum contains anti-human cell Abs and is being used to activate human complement through the classical pathway on various human tumor and nontumoral cells, including HeLa and HEK 293T cells. Mouse anti-p-JNK and rabbit anti-p65 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-JNK Abs were purchased from Cell Signaling Technologies (Beverly, MA). Mouse anti-actin Abs were purchased from Chemicon (Temecula, CA). Abs directed to human C3 were prepared in a goat and were shown by Western blotting to bind primarily to C3 (α- and β-chains) in human serum. Mouse Abs directed to a neoepitope in human C5b-9 (clone aE11) was purchased from Dako (Carpinteria, CA). The following Abs directed to human membrane complement regulatory proteins were used: anti-CD46 (clone GB24; provided by Dr. John Atkinson, St. Louis, MO), anti-CD55 purchased from Hycult Biotech (Uden, The Netherlands), and anti-hCD59 (clone BRIC229) purchased from the International Blood Group Reference Laboratory (Birmingham, U.K.). Anti-mouse CD59a was provided by Dr. Paul Morgan (University of Wales College of Medicine, Cardiff, U.K.). Monoclonal rat anti-mouse Crry was purchased from BD Biosciences. FITC-conjugated Abs directed to mouse, rabbit, goat, and rat IgG and peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased form Jackson ImmunoResearch Laboratories (West Grove, PA). PS-1145 (an IKK inhibitor), propidium iodide (PI), streptolysin O (SLO), melittin, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO).

**Cytotoxicity assays**

Cell lysis by complement (NHS) was measured by the PI and trypan blue inclusion methods as described before (6). Both methods measure plasma membrane damage and permeability and were used interchangeably. Briefly, human cells were treated with rabbit anti-K562 Abs for 30 min on ice followed by treatment with 50% NHS for 60 min at 37˚C. Mouse cells were sensitive to 4–15% NHS and did not require pretreatment with...
abs, probably due to presence of non-specific mouse-anti Abs in NHS. NHS lost its cytotoxic activity on mouse cells after IgG depletion (M. Masarwa and Z. Fishelson, unpublished observations). Human complement was used to treat mouse fibroblasts since mouse complement has a lower cytotoxicity and is labile. Quantitative but not qualitative differences have been reported between cytotoxicity of C5b-9 complexes from various animal species. For the PI inclusion assay, PI was added to a final concentration of 0.5 µg/ml and EDTA to 1.5 mM. Cells were kept on ice and immediately examined in a FACScan (BD Biosciences). The data were analyzed by using WinMDI 2.8 and the percentage of dead (PI+) cells was calculated. Trypan blue inclusion was counted microscopically after addition of 0.2% trypan blue to the cells on ice. Statistical significance was analyzed by using the two-sided unpaired Student t test. All experiments included negative controls that tested the baseline death level. Death of control cells treated either without NHS (0% NHS) or with HIS (at the same concentration as NHS) was also determined.

Lysis of cells by SLO and melittin

HeLa cells (1 × 10⁴) treated or not with p65 small interfering RNA (siRNA) for 2 d, as described below, were incubated for 10 min at 37°C with SLO and preactivated for 5 min at room temperature with 10 mM DTT or with DTT alone as control. FCS (final 5%) was added and incubation was continued for an additional 50 min at 37°C. Cells were also treated with the bee venom polypeptide melittin for 60 min at 37°C. Percentage lysis was determined as described above.

RNA interference, transient plasmid transfection, and confocal microscopy

Cells (3 × 10⁴ per well) in a 24-well plate (Corning) were transfected with 60 pmol p65-specific RNA duplex (Stealth siRNA: Invitrogen), JNK1-specific RNA duplex (sense, 5'-GACCAUUUGCAGAUCGACU-3'; Dharmacon), JNK2-specific RNA duplex (sense, 5'-GAUGCUAAACUGUGCAUU-3'; Dharmacon), or with a nonspecific control RNA duplex, mixed with Oligofectamine, according to the manufacturer’s instructions. HeLa cells (20 × 10⁴ per well, in six-well plate (Corning)) were transfected with 300 pmol p65-specific RNA duplex or with a nonspecific control RNA duplex, mixed with Oligofectamine. The cells were kept in the incubator for 2 d for maximal silencing before analysis.

pEGFP-p65 was provided by Dr. Lienhard Schmitz (University of Giessen, Giessen, Germany). p65⁻/⁻ MEFs were transfected with plasmid DNA mixed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. HEK 293T cells were transfected by using the calcium phosphate method, as previously described (35). Two days after transfection, the cells were treated or not and then fixed with 1% formaldehyde. Enhanced GFP (EGFP) fluorescence in cells was viewed by confocal microscopy in a Leica SP2 laser confocal microscope (Leica Microsystems, Wetzlar, Germany).

Analysis of cell lysates by Western blotting

Cells were solubilized with cell lysis buffer (50 mM β-glycerophosphate [pH 7.3], 1% Triton X-100, 1.5 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 1 mM DTT, 0.1 mM sodium orthovanadate, protease inhibitor mixture (Sigma-Aldrich), and phosphatase inhibitor mixture (Sigma-Aldrich)) and cleared by centrifugation at 20,000 × g for 15 min at 4°C. Protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL). Proteins were subjected to SDS-polyacrylamide gel (10%) electrophoresis under reducing conditions (50 mM DTT) and transferred onto a nitrocellulose membrane (Schleicher & Schuell Bio-Membranes). The membrane was blocked with 5% skim milk (Truva, Rehovot, Israel) in TBS containing 0.05% Tween 20 (Sigma-Aldrich) for 1 h at room temperature. The membrane was treated with rabbit anti-p65 (1:1000), mouse anti-iKbα (1:1000), or rabbit anti-iKbβ (1:1000) Abs overnight at 4°C or mouse anti-Actin Abs for 1 h at room temperature and then with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG for 1 h at room temperature. Bands were developed with an ECL substrate (Pierce) and exposed to a Super RX film (Fuji, Tokyo, Japan).

Analysis of surface C3, C5b-9, and complement regulators by flow cytometry

To activate C3 deposition, mouse fibroblasts were treated with 10% CD5-/-HS or HIS for 15 min at 37°C and HeLa cells were treated with Ab followed by 10% CD5-/-HS or HIS for 15 min at 37°C. To activate C5b-9 deposition, the cells were treated as above, but with 10% NHS or HIS. The cells were then washed with PBS and treated with goat anti-human C3 antisera or mouse anti-neo C5b-9 (clone aE11) mAb for 30 min at 4°C, washed again, and labeled with FITC-conjugated secondary Abs. To determine the level of expression of complement regulators, mouse fibroblasts were treated with rabbit anti-mouse CD59a (diluted 1:100) or rat anti-mouse Cry (diluted 1:150) and HeLa cells were treated with mouse anti-human CD46 (diluted 1:50), mouse anti-human CD55 (diluted 1:50), or mouse anti-human CD59 (diluted 1:100) for 30 min at 4°C. After washing, the cells were labeled with FITC-conjugated secondary Abs. Labeled cells were examined by flow cytometry in a FACScan. Mean fluorescence intensity (MFI, G-mean) values for 7000 cells were analyzed by using WinMDI 2.8.

Results

NF-κB protects cells from CDC

NF-κB is a major regulator at the crossroad between life and death with known antiapoptotic activities (36). The role of NF-κB in complement-mediated cell death was studied first in MEFs lacking the p65 subunit of NF-κB (p65⁻/⁻) or lacking the iKbα subunit or iKbβ subunit of the NF-κB activator complex (iKk). WT, p65⁻/⁻, and iKk⁻/⁻ MEFs were reacted with complement and cell lysis was quantified. Compared to WT MEFs, p65⁻/⁻ MEFs, iKkα⁻/⁻ MEFs, and iKkβ⁻/⁻ MEFs were significantly more sensitive to complement-mediated necrotic cell death (Fig. 1A,
Next, the effect of p65 silencing on complement-mediated death was examined in HeLa cells that underwent good transfection and silencing, thus also expanding the range of tested cells and excluding description of a cell-specific phenomenon. Cells were transfected with p65 siRNA or with a nonspecific control siRNA. Silencing efficiency of p65 siRNA was assessed 48 h after transfection by Western blotting using anti-p65 and anti-actin Abs (Fig 1C, right panel). Cells were also subjected to treatment with Ab and NHS and percentage cell death was determined by PI inclusion (Fig. 1C). Whereas control siRNA had no effect on HeLa complement sensitivity, compared with untransfected cells, p65 silencing sensitized cells to complement-mediated necrotic cell death.

Cells lacking the IKKα or IKKβ subunit of the NF-κB activator complex are more sensitive to CDC than are WT cells (Fig. 1B), suggesting involvement of IKK in complement resistance. PS-1145 specifically blocks NF-κB activation by inhibiting phosphorylation of IκBα, the key negative regulator of p65 activity, by IKK (37). The effect of short-term inhibition of the NF-κB pathway with PS-1145 on CDC was examined. Pretreatment with PS-1145 enhanced complement-mediated cell death of WT MEFs. In contrast, PS-1145 had no effect on death of p65<sup>-/-</sup> MEFs, further supporting a role for IKK and the p65 pathway in cell resistance to CDC.

Complement-mediated death of p65<sup>-/-</sup> MEFs reconstituted with plasmid p65 was examined. p65<sup>-/-</sup> MEFs were transiently transfected with increasing quantities of pEGFP-p65 plasmid (1–3 µg). As shown in Fig. 2A, increasing doses of recombinant p65-EGFP were synthesized by the transfected cells. Restoration of p65 in p65<sup>-/-</sup> cells correlated with a dose-dependent reduction of sensitivity to complement-mediated cell death (Fig. 2B).

To examine whether NF-κB protects cells from pore formers in general or is selective to C5b-9, the impact of p65 silencing on cell death inflicted by the pore formers SLO and melittin was tested. SLO and melittin use a similar mechanism to that of complement MAC to incorporate into cell membranes, induce Ca<sup>2+</sup> influx, and produce cell death (38). HeLa cells were transfected, using Oligofectamine, with p65 siRNA or with a nonspecific control siRNA. Forty-eight hours after transfection, untransfected and transfected cells were treated with SLO preactivated with DTT or DTT alone as control or with melittin. As shown in Fig. 3, p65 silencing by siRNA had no effect on cell death mediated by melittin (Fig. 3A) or SLO (Fig. 3B).

**NF-κB does not protect cells from other pore formers**

FIGURE 3. Cell death by SLO and melittin is not regulated by p65. HeLa cells were transfected, using Oligofectamine, with 60 pmol p65 siRNA or with a nonspecific control siRNA. Forty-eight hours after transfection, the cells were treated with melittin (A) or with SLO (pre-activated for 5 min at room temperature with 10 mM DTT) or with DTT alone (B) for 60 min at 37°C. Percentage necrotic cell death was determined by trypan blue inclusion. Results of three independent experiments are expressed as the mean percentage cell death ± SD. NT, Non-transfected.

FIGURE 4. Complement regulators and complement activation on p65-deficient cells. (A) WT and p65<sup>-/-</sup> MEFs were labeled with anti-mCD59 or anti-Cry Abs and with secondary FITC-conjugated Abs and then analyzed by flow cytometry. (B) Complement C3 and C5b-9 deposition on p65<sup>-/-</sup> MEFs was also examined in WT and p65<sup>-/-</sup> MEFs treated with C8-deficient human serum or NHS (10%), respectively, for 15 min at 37°C. The cells were washed, treated with anti-C3 or anti-C5b-9 aE11 Abs and FITC-labeled second Abs, and analyzed by flow cytometry. (C) HeLa cells were transfected with p65-specific siRNA or control siRNA. After 48 h the cells were incubated with anti-human CD59, CD46, or CD55 Abs and with 10% NHS. Percentage necrotic cell death was determined by trypan blue inclusion. Results of three independent experiments are expressed as the mean percentage cell death ± SD. NT, Non-transfected.
Effect of NF-κB on complement deposition

NF-κB regulates transcription of numerous target genes (39). Therefore, NF-κB knockdown could potentially modify cell surface proteins, leading to enhanced complement activation and increased deposition of complement proteins on the cells. The extent of complement C5b-9 deposition and cell death does not correlate well with quantity of Ab binding, but it correlates excellently with the level of expression of the membrane complement regulators (Ref. 12 and Z. Fishelson, unpublished data). Thus, for example, a decrease in the level of expression of the membrane complement regulator CD59 may lead to enhanced complement deposition and cell death. The level of expression of murine Crry and CD59a on WT and p65−/− MEFs was measured by flow cytometry, as described in Materials and Methods. The levels of Crry and CD59 on p65−/− were somewhat lower than on WT MEFs (Fig. 4A). Analysis of the level of expression of CD46, CD55, and CD59 on HeLa cells transfected with p65 siRNA or control siRNA showed no difference in expression of these regulatory proteins (Fig. 4C). The relative capacity of WT and p65−/− cells to activate complement was also compared. WT and p65−/− MEFs were treated with C8D-HS for C3 deposition or NHS for C5b-9 deposition as described in Materials and Methods. Cells were washed and labeled with anti-C3 or anti–C5b-9 aE11 Abs followed by FITC-labeled second Abs. The lower membrane regulator expression in the absence of p65 had no impact on C3 and C5b-9 deposition on MEFs (Fig. 4B). Similarly, HeLa cells transfected with p65 siRNA or control siRNA were treated with Ab and NHS and analyzed for C3 and C5b-9 deposition. C3 deposition was not affected by p65 silencing whereas a small increase of MAC deposition was observed on HeLa cells transfected with p65 siRNA relative to control siRNA (Fig. 4D).

Is NF-κB targeting JNK in its protective activity from complement?

JNK activation is involved in cell death induced by complement (7). To examine the hypothesis that NF-κB protects cells from complement by downregulating the JNK pathway, the effect of NF-κB silencing on death of cells lacking p65 was investigated. HeLa cells were transfected with p65 siRNA, JNK siRNA, or both. Silencing of p65 and JNK was validated 48 h after transfection by Western blotting, using anti-p65, anti-JNK, and anti-actin Abs (Fig. 5B). At the same time, these transfectants were subjected to CDC by Ab and NHS. As shown in Fig. 5A and as expected, p65 silencing enhanced cell death whereas JNK silencing reduced cell death. Interestingly, p65 silencing lost its enhancing effect in JNK−/− cells. This suggests that p65 has no protective effect in the absence of JNK.

To further demonstrate an effect of NF-κB on JNK activation, the kinetics of complement-induced JNK activation were examined in WT and p65−/− MEFs. Cells were treated with NHS or HIS for 10–30 min at 37 °C and cell extracts were subjected to Western blotting with anti–p-JNK or anti-actin Abs. HIS induced in both cell lines low-level JNK activation, probably due to serum effects (Fig. 5C, 5D). In contrast, following sublytic complement (HIS) treatment, JNK activation was significantly higher in p65−/− MEFs (Fig. 5D) than in WT MEFs (Fig. 5C).

**FIGURE 5.** Crosstalk between p65 and JNK in regulation of CDC. (A and B) HeLa cells were transfected with p65 siRNA, JNK siRNA, with both siRNAs, or with a nonspecific control siRNA. Forty-eight hours after transfection, the cells were treated with Ab and NHS (50%) for 60 min at 37 °C. Percentage necrotic cell death was determined by trypan blue inclusion. Results of three experiments are expressed as the mean percentage cell death ± SD. *p < 0.05 relative to control siRNA. (B) Transfected HeLa cells in (A) were extracted and examined by Western blotting with anti-p65, anti-JNK, or anti-actin Abs. Anti-JNK Ab detects both p54 and p46 JNK isoforms. (C and D) WT (C) and p65−/− (D) MEFs were treated with 7% NHS or HIS at 37 °C for the indicated times. Cell extracts were subjected to Western blotting with anti–p-JNK (and anti-actin) Abs. Band intensities of the p54 JNK isoform were quantified by densitometry and corrected according to the intensity of the actin band. In three independent experiments, the corrected optical densities of the p-JNK bands were adjusted relative to the p-JNK band of HIS-treated cells at the 10 min point, which was set as 100. The mean adjusted p-JNK band intensities of the three experiments ± SD are shown. *p < 0.05 relative to HIS.
Discussion

The cell death and survival signals that activate and regulate complement-dependent cytotoxicity mediated by the complement C5b-9 complex are not well characterized. At least to some extent, the necrotic cell death inflicted by the C5b-9 complex is programmed and involves Bid and JNK (6, 7). Some of the death signals are counteracted by ERK (9). Results presented in this study demonstrate that the NF-κB signaling pathway also participates in cell protection from complement-mediated cell death. This claim is supported by several findings: 1) cells lacking either the p65 subunit of NF-κB or the IKKα or IKKβ subunits of the NF-κB activator complex are more sensitive to CDC; 2) knockdown of p65 with siRNA renders cells more susceptible to CDC; 3) pretreatment of target cells with the IKK inhibitor PS-1145 increases their sensitivity to CDC, but only in p65-deficient cells; and 4) reconstitution of p65 into p65−/− cells or overexpression of p65 in p65-deficient cells enhances their resistance to CDC. Apparently, both p65 and IKKβ of the canonical NF-κB pathway and IKKα of the noncanonical NF-κB pathway are involved in the protection from CDC. One possibility is that both canonical and noncanonical NF-κB pathways promote resistance to CDC. Alternatively, the canonical and noncanonical NF-κB signaling pathways may cross-regulate each other and act as a single NF-κB signaling system in promotion of complement resistance (reviewed in Refs. 24, 40). It is also conceivable that IKKα supports activity of IKKβ in the conventional signaling pathway by facilitating assembly of the holo-IKK complex. IKKs also have NF-κB–independent functions (41). Because IKKα and IKKβ knockout cells were persistently more sensitive to CDC than were p65 knockout cells, it is suggested that IKKα and IKKβ might also have NF-κB–independent effects on complement resistance. This remains to be further elucidated.

Cell death induced by lytic doses of the C5b-9 complexes is very fast and is irreversible within 10–30 min. Therefore, the likelihood of coincidental induction of elevated complement resistance with C5b-9–induced NF-κB transcriptional activities is slim. Unlike treatment with TNF-α, treatment with sublytic complement for 15–60 min does not activate nuclear translocation of p65 in HEK 293T cells (Supplemental Fig. 1). We suggest that constitutively active NF-κB transcribes synthesis of proteins that assist in elimination of the C5b-9 complexes or counteract the damaging effect inflicted by the C5b-9 complexes. Sublytic C5b-9–induced NF-κB activation was demonstrated in human vascular smooth muscle cells (3), rat glomerular epithelial cells (42), and HUVECs (43), but not in rat glomerular mesangial cells (44). In those studies, the time required for NF-κB activation by sublytic C5b-9 was 30 min to 5 h. It is conceivable that under sublytic conditions and longer treatment of cells such as vascular smooth muscle cells and glomerular epithelial cells, C5b-9–induced NF-κB–regulated proteins may further enhance complement resistance.

Which of the numerous genes targeted by the NF-κB homo- and heterodimers confers resistance to CDC is still not known. Some light may be shed by the result showing that the increase in complement sensitivity of cells treated with a p65 siRNA is abrogated upon silencing of JNK (Fig. 4). This suggests that the fate of cells subjected to CDC is determined by a crosstalk between the NF-κB and JNK pathways. One possibility is that a constitutively synthesized phosphatase regulated by NF-κB is regulating JNK. Such a phosphatase, by directly or indirectly inactivating JNK, may protect cells from CDC by inhibiting the JNK lytic activity. In support of this possibility, our results show that complement-induced JNK phosphorylation is elevated in cells deficient in p65 (Fig. 5). Crosstalk between NF-κB and JNK has been previously reported (reviewed in Ref. 45). TNF-α–induced JNK activation is prolonged in cells expressing a degradation-resistant IkBα and in cells treated with the phosphatase inhibitor vanadate (30). Gadd45β is induced by NF-κB and blocks the proapoptotic JNK pathway (29) by inhibiting MKK7 (46).

Interestingly, NF-κB p65 protects cells from the complement membrane attack complex but not from other pore formers, such as SLO and melittin (Fig. 3). This indicates that regulation of necrotic cell death induced by C5b-9 and SLO has distinct characteristics. The first line of defense from complement depends on the membrane complement regulators (10). Less CD59 and Crry are expressed on mouse embryonic fibroblasts lacking p65 than on WT cells, yet similar levels of C3 and C5b-9 are deposited on p65-deficient and p65–sufficient cells (Fig. 4). The same level of CD46, CD55, and CD59 is expressed on HeLa cells transfected either with p65 siRNA or control siRNA, but slightly more C5b-9 (but not C3) binds to HeLa cells with knocked down p65. Thus, the data do not show any clear correlation between the elevated sensitivity of NF-κB–deficient cells to CDC and reduced expression of membrane complement regulators leading to an enhanced deposition of complement C3 and C5b-9 complexes on the cells. Because the regulation of C5b-9 formation and stability is multifactorial and depends, besides the membrane complement regulators, on soluble complement regulators such as factor H and C4b binding protein, on cell surface sialic acid residues, and on several cellular mechanisms (reviewed in Ref. 10), this is not surprising. Bid is involved in necrotic cell death activated by both C5b-9 and SLO (6). Unlike C5b-9, SLO does not induce JNK activation (7). Hence, it is suggested that JNK activation is involved in CDC but not in cell death induced by SLO or melittin. Consequently, the NF-κB p65–dependent protective mechanism targeting JNK would be effective against CDC but not against cell death induced by SLO and melittin. Furthermore, C5b-9 activates necrotic cell death via JNK-dependent and JNK-independent pathways (7). That knockdown of p65 has no effect on JNK−/− cells (Fig. 5A) suggests that p65 has no protective effect in the JNK-independent lytic pathway activated by C5b-9.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


A role for the NF-κB pathway in cell protection from complement-dependent cytotoxicity

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Supplemental data

Fig. 1S. Treatment of HEK 293T cells with sublytic complement for 15-60 minutes does not induce nuclear translocation of EGFP-p65. 293T cells were transfected with pEGFP-p65 plasmid DNA (3 μg). After 48 h, the ability of EGFP-p65 to translocate into the nucleus upon activation by TNFα was examined by confocal fluorescence microscopy. The cells were incubated at 37°C with TNFα 4u/μl for 60 min. As expected, most of the EGFP-p65 translocated into the nuclei of TNFα-treated 293T cells (A). Transfected cells were also treated with sublytic antibody and HIS or NHS (50%) for 15-60 min at 37°C and EGFP-p65 location was viewed under a confocal microscope. Representative pictures of cells treated for 30 min with HIS (B) or NHS (C) are shown. No nuclear translocation of EGFP-p65 was observed in 293T cells treated for 15-60 min with complement.

Gancz et al.- Fig. 1S