# cFLIP Protein Prevents Tumor Necrosis Factor-α–Mediated Induction of Caspase-8–Dependent Apoptosis in Insulin-Secreting βTc-Tet Cells

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**Type 1 diabetes is characterized by the infiltration of activated leukocytes within the pancreatic islets, leading to β-cell dysfunction and destruction. The exact role played by interferon-γ, tumor necrosis factor (TNF)-α, and interleukin-1β in this pathogenic process is still only partially understood. To study cytokine action at the cellular level, we are working with the highly differentiated insulin-secreting cell line, βTc-Tet. We previously reported that it was susceptible to apoptosis induced by TNF-α, in combination with interleukin-1β and interferon-γ. Here, we report that cytokine-induced apoptosis was correlated with the activation of caspase-8. We show that in βTc-Tet cells, overexpression of cFLIP, the cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein, completely abolished cytokine-dependent activation of caspase-8 and protected the cells against apoptosis. Furthermore, cFLIP overexpression increased the basal and interleukin-1β–mediated transcriptional activity of nuclear factor (NF)-κB, whereas it did not change cytokine-induced inducible nitric oxide synthase gene transcription and nitric oxide secretion. The presence of cFLIP prevented the weak TNF-α–induced reduction in cellular insulin content and secretion; however, it did not prevent the decrease in glucose-stimulated insulin secretion induced by the combined cytokines, in agreement with our previous data demonstrating that interferon-γ alone could induce these β-cell dysfunctions. Together, our data demonstrate that overexpression of cFLIP protects mouse β-cells against TNF-α–induced caspase-8 activation and apoptosis and is correlated with enhanced NF-κB transcriptional activity, suggesting that cFLIP may have an impact on the outcome of death receptor–triggered responses by directing the intracellular signals from β-cell death to β-cell survival. *Diabetes* 51:1805–1814, 2002**

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cFLIP, cellular FLICE-inhibitory protein; CHX, cycloheximide; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; ELISA, enzyme-linked immunosorbent assay; FADD: Fas-associated death domain protein; FLAG, octapeptide tag; FLICE: FADD-like interleukin-1β–converting enzyme; GSIS, glucose-stimulated insulin secretion; IAP, inhibitor of apoptosis; IBMX, isobutylmethylxanthine; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; KRBH, HEPES-buffered Krebs-Ringer bicarbonate buffer; NF, nuclear factor; PGK, phosphoglycerate kinase; *p*NA, *p*-nitroanilide; RIP, receptor-interacting protein; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; TNFR1, TNF-α receptor 1; TRADD, TNF receptor–associated death-domain protein; TRAF, TNF receptor–associated factor;

Pathogenesis of type 1 diabetes is characterized by the progressive appearance of a lymphocytic infiltrate, termed insulitis, in the pancreatic islets of Langerhans. Upon interaction with β-cell autoantigens, activated leukocytes secrete the cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β, which can induce dysfunction and destruction of the insulin-secreting β-cells, resulting in insulin deficiency and hyperglycemia (1). These inflammatory mediators have been found in the insulitis of NOD (nonobese diabetic) mice and in the pancreas of type 1 diabetic patients. They are involved in both direct effects through control of gene expression in β-cells (2) and indirect effects through activation of immune and inflammatory cells present within the islets. Their exact role in the pathogenesis of type 1 diabetes, however, is not firmly established (3,4).

Even though the direct effects of TNF-α in the progression to diabetes remain unclear, there is evidence that this cytokine can mediate the destruction of β-cells (5). Whereas TNF-α alone leads to apoptosis of the mouse pancreatic cell line NIT-1, cytokine-induced cell death of mouse primary β-cells relies on the combined action of TNF-α and IFN-γ (6). Another reported effect of TNF-α on β-cells is the inhibition of glucose-stimulated insulin secretion (GSIS). Indeed, when exposed to high concentrations of TNF-α, insulin secretion in murine islets has been shown to be impaired, an effect enhanced by addition of IFN-γ (7). In contrast, only low amounts of TNF-α are sufficient to reduce the secretory activity of rat islets, reflecting species-specific regulation of β-cell function by TNF-α (8).

The role of TNF-α in mediating β-cell dysfunction and destruction is still unclear. Indeed, treatment of NOD mice with TNF-α may either prevent or exacerbate the disease, depending on the age of the mice. For instance, islet-restricted expression of TNF-α in neonatal NOD mice resulted in accelerated development of diabetes (9), whereas NOD mice expressing the transgene later in life were protected from diabetes (10). Kagi et al. (11) also reported that NOD mice lacking TNF-α receptor 1 (TNFR1) were protected from diabetes.

Death receptors belonging to the TNF receptor gene superfamily, such as TNFR1 (p55/CD120a) and Fas (CD95/Apo1), are defined by similar, cysteine-rich extracellular

domains and by homologous cytoplasmic sequences termed death domains (DDs) that are essential for cell death signaling. TNF-α as well as Fas ligand are predicted to exist as trimers, and ligand binding leads to clustering of the receptor molecules and initiation of signaling (12). The TNF-α signal transduction pathway not only mediates cell death, but also directs gene expression by activating the transcription factor nuclear factor (NF)-κB. In the apoptotic response, the DDs of TNFR1 bind to the adapter protein TRADD (TNF receptor–associated DD protein) (13), which then binds FADD (Fas-associated DD protein) (14–16). FADD then recruits caspase-8 (FADD-like interleukin-1β–converting enzyme [FLICE]/MACH) (17,18) by interaction with protein death effector domains (DEDs), thus forming a death-inducing signaling complex (DISC) (19,20). Cleavage of pro-caspase-8 allows the release of activated caspase-8 and the initiation of the apoptotic response by cleavage of downstream effector caspases, among them caspase-3, -6, and -7. On the other hand, TRADD associated with TNFR1 can recruit RIP (receptor-interacting protein) (21,22) and TRAF2 (TNF receptor–associated factor 2) (23) to initiate a signaling cascade leading to the activation and translocation of NF-κB to the nucleus. This promotes the expression of several genes including those coding for anti-apoptotic proteins such as A20 (24), the inhibitor of apoptosis (IAP) proteins c-IAP-1 and -2 (25), TRAF-1/-2 (26), and Bcl-$X_L$ (27).

A number of inhibitors of the TNF-α apoptotic pathway have been described, among them the FLICE-inhibitory proteins, identified in viruses (vFLIP) (28) and mammalian cells (cFLIP; also called FLAME-1, I-FLICE, CASH, Casper, CLARP, MRIT, or usurpin) (29–36). cFLIP contains two DEDs and a caspase-like domain with significant homology to caspase-8 but lacks any proteolytic activity. It is thought that cFLIP blocks the apoptotic cascade through its binding to FADD via the DEDs (29,32), thus preventing the recruitment of caspase-8 into the DISC. Overexpression of cFLIP may thus divert the TNF-α signaling pathway to activation of NF-kB and induction of anti-apoptotic genes, since TNFR1-TRADD interactions are not blocked by cFLIP (37).

To evaluate the potential of interfering with the TNF-α pathway by genetic modification of β-cells, and the consequences of this inhibition on cytokine-induced caspase activation and apoptosis, NF-κB activity, NO production, and impaired GSIS, we used the conditionally immortalized βTc-Tet cells. These cells display normal GSIS, can be growth-arrested in the presence of tetracyclin, and, when transplanted in diabetic syngeneic mice, can maintain normoglycemia for several months (38). We previously demonstrated that Bcl-2 overexpression in these cells, referred as to CDM3D cells, improved resistance against stress-induced apoptosis and increased viability at high cell density (39). In studies to assess the effects of interfering with either the IL-1β– or IFN-γ–induced signaling pathway in β-cells, CDM3D cells were further engineered to overexpress dominant-negative mutants of MyD88 (MyD88Toll, MyD88lpr) (40) or suppressor of cytokine signaling (SOCS)-1 (41), respectively. Both MyD88Toll/lpr CDM3D and SOCS-1 CDM3D cells displayed suppression of IL-1β/IFN-γ–induced inducible nitric oxide synthase (iNOS)/nitric oxide (NO) upregulation as well as increased resistance to cytokine-induced apoptosis. Unlike cells expressing SOCS-1, however, MyD88Toll/lpr CDM3D cells were not protected against impaired GSIS induced by cytokines.

Here we demonstrate that the stable expression of cFLIP in CDM3D cells protects mouse βTc-Tet cells against cytokine-induced caspase-8 activation and apoptosis. Furthermore, the stable expression of cFLIP results in

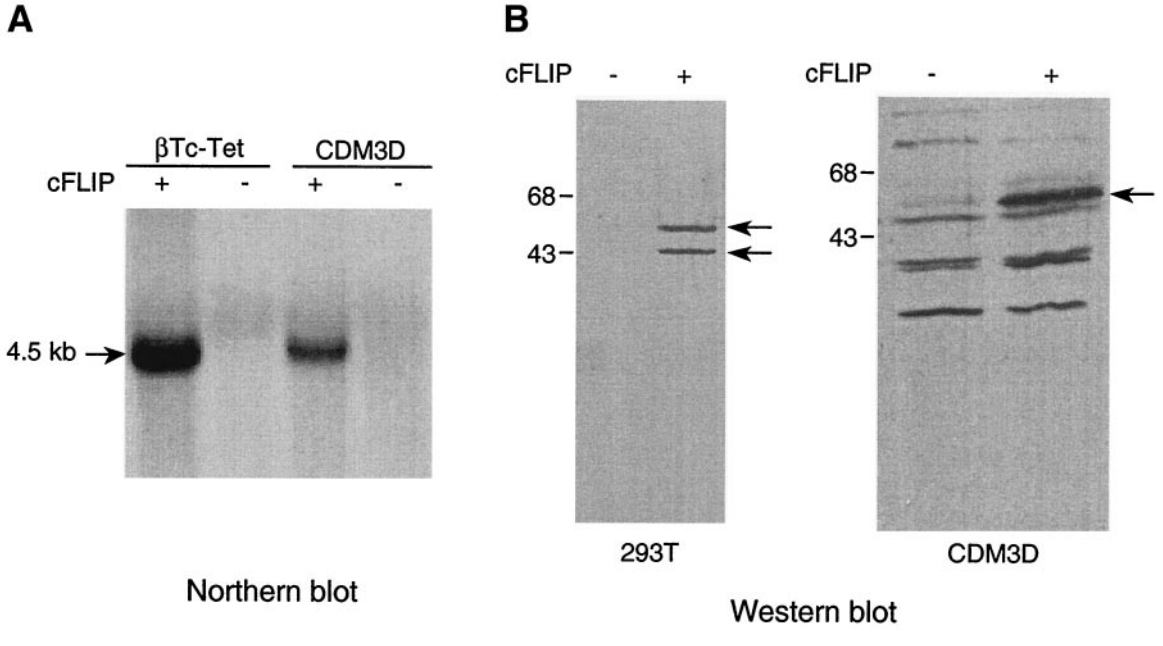
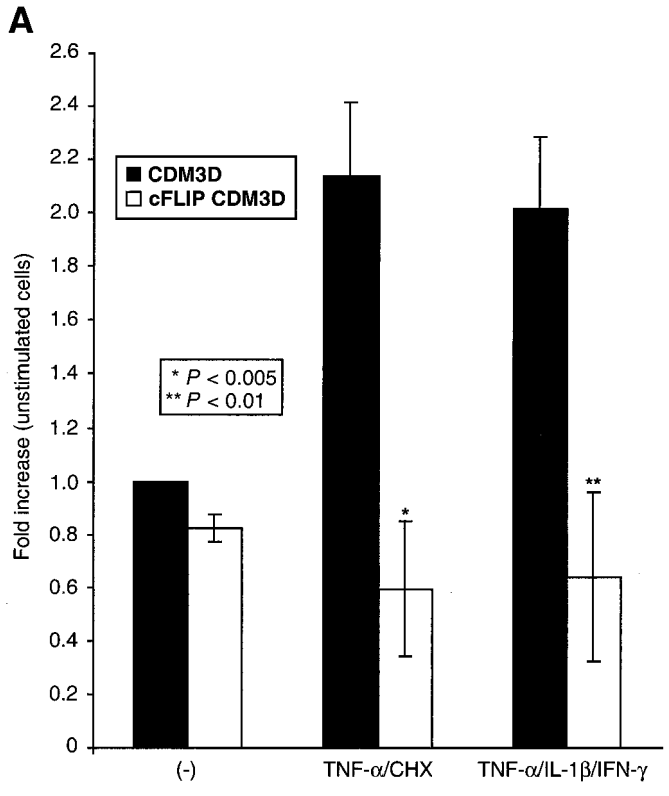
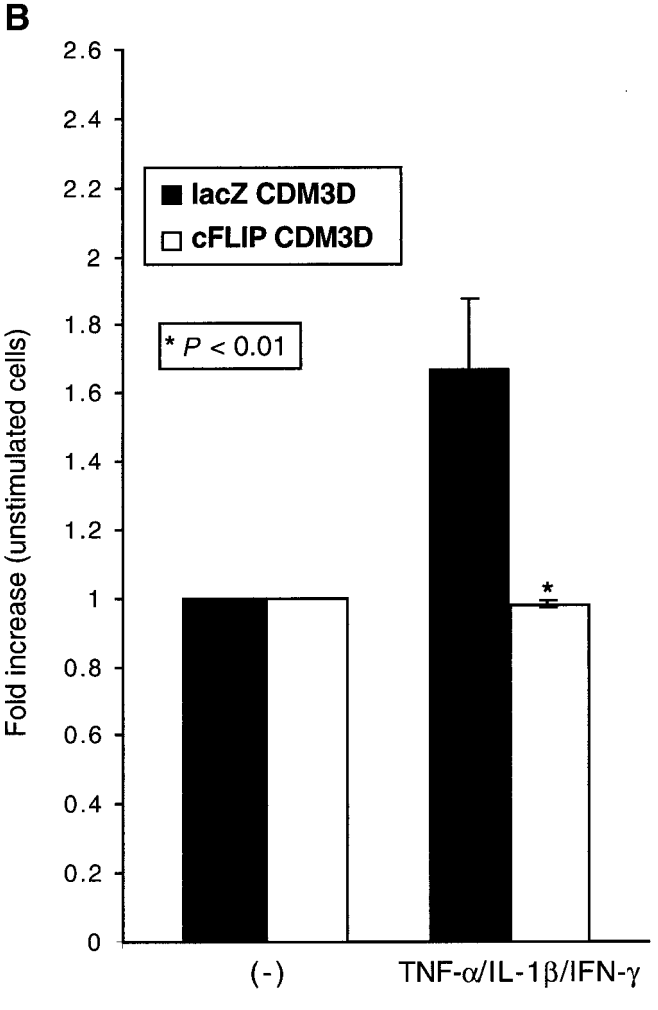



**FIG. 1. Expression of human cFLIP mRNA and protein in CDM3D cells. *A*: Detection of cFLIP mRNA by Northern blot analysis. A 4.5-kb band corresponding to cFLIP transcript was detected in transduced (+) but not in nontransduced (–) βTc-Tet and CDM3D cells. *B*: The lysates of control (–) and transduced (+) cells were analyzed by Western blotting. In 293T cells, cFLIP appeared as a full-length recombinant protein of 55 kDa and as a 43-kDa band corresponding to a COOH-terminally truncated protein. In cFLIP-transduced CDM3D cells, only the full-length cFLIP was observed. The data are representative of three independent experiments.**



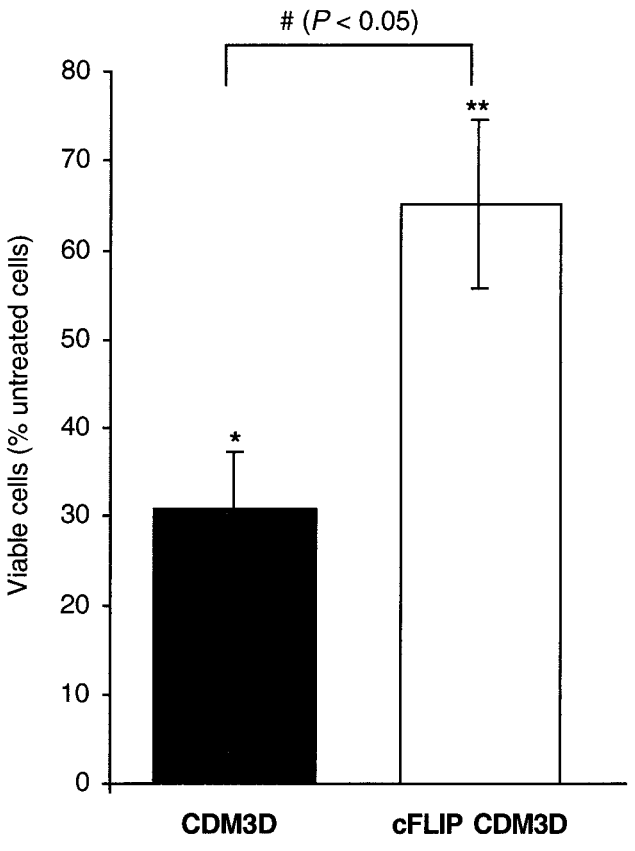




**FIG. 3. Recombinant cFLIP protects against cytokine-induced apoptosis. CDM3D or cFLIP CDM3D cells were exposed for 36 h to TNF-α, IL-1β, and IFN-γ ($10^3$ units/ml each), and the percentage of viable cells was determined. The results showed that cFLIP protected cells against cytokine-induced apoptosis. Data are means ± SE of three independent experiments, each performed in triplicate. *$P < 0.0001$, **$P < 0.005$ vs. untreated cells; #$P < 0.05$ vs. cytokine-treated CDM3D cells.**

an increase in the basal and cytokine-induced transcriptional activity of NF-κB. In contrast, recombinant cFLIP does not affect the ability of CDM3D cells to induce iNOS gene transcription and NO production in response to IL-1β and IFN-γ. Reduction in GSIS induced by TNF-α–treated cells was prevented by cFLIP, but not that induced by the three cytokines, in agreement with the major role of IFN-γ in this inhibitory process. This study clarifies the mode of action of TNF-α in βTc-Tet cells and proposes a genetic way to interfere with its intracellular signaling pathway.

## RESEARCH DESIGN AND METHODS

**Cell culture.** CDM3D cells are βTc-Tet cells (38) that have been modified to overexpress Bcl-2 (39). They were grown in Dulbecco's modified Eagle's medium (Life Technologies, Basel, Switzerland) containing 25 mmol/l glucose and supplemented with 15% horse serum (Amimed; BioConcept, Allschwil,

**FIG. 2. Cytokine-dependent activation of caspase-8 is inhibited by expression of cFLIP. Cells were not treated (–) or treated for 5 h with TNF-α ($10^3$ units/ml) plus CHX (0.5 μg/ml) or a combination of the three cytokines (TNF-α, IL-1β, and IFN-γ) at $10^3$ units/ml each. The activity of caspase-8 was assessed by incubating cell lysates for 2 h in the presence of 200 μmol/l of the protease substrate IETD-pNA before reading the absorbance at 405 nm. *A*: Activation of caspase-8 upon cytokine exposure was completely abolished in CDM3D cells expressing cFLIP. Data are means ± SE; $n = 7$. *$P < 0.005$, **$P < 0.01$ vs. CDM3D cells. *B*: Activation of caspase-8 upon cytokine treatment of CDM3D cells transduced with a control gene (lacZ) but not in cFLIP-transduced cells. Data are means ± SE; $n = 3$. *$P < 0.01$ vs. lacZ CDM3D cells.**

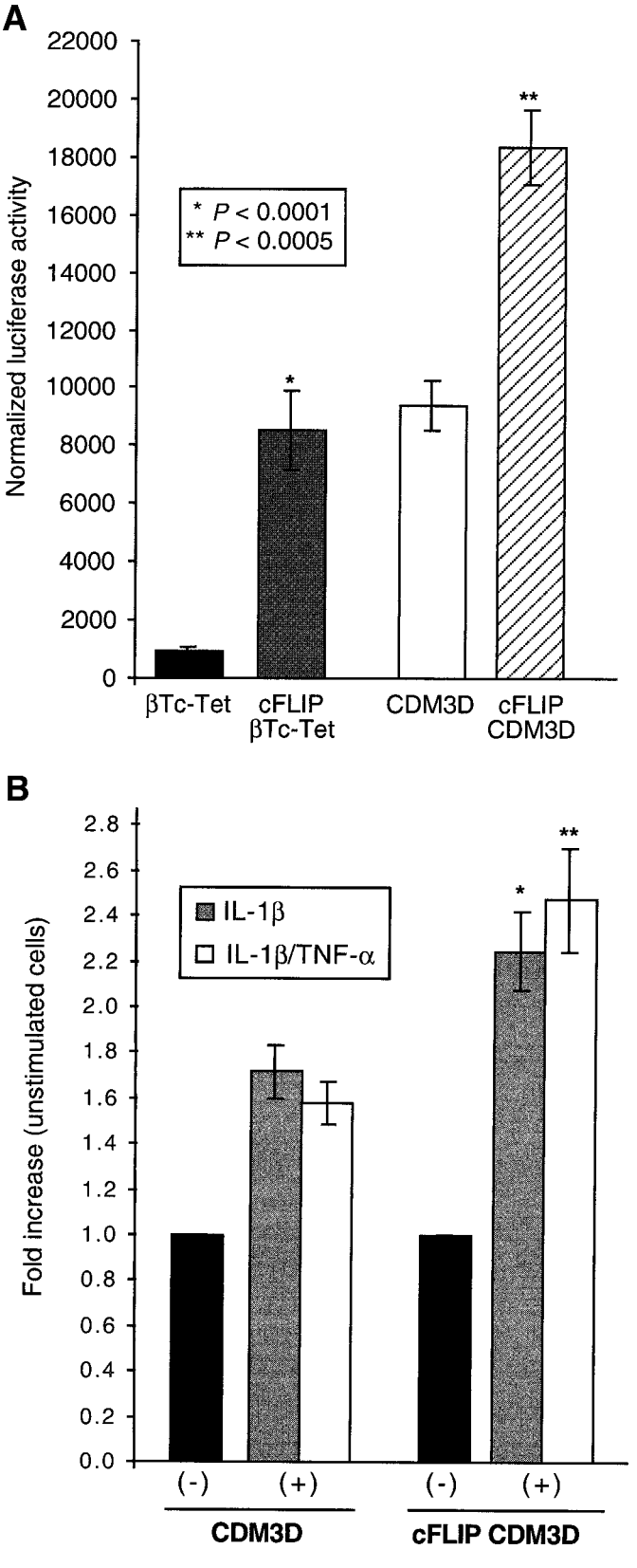



**FIG. 4. Stable expression of cFLIP enhances NF-κB basal and cytokine-induced transcriptional activity. βTc-Tet and CDM3D cells transduced or not with cFLIP were cotransfected with a NF-κB-luciferase reporter and a control cytomegalovirus-LacZ construct. Seventy-two hours after transfection, luciferase and β-galactosidase activities were measured; data are expressed as luciferase activity normalized to that of β-galactosidase. *A*: In the presence of cFLIP protein, the basal level of NF-κB reporter activity was increased; also, expression of Bcl-2 in βTc-Tet cells (CDM3D cells) increased NF-κB transcriptional activity. Data are means ± SE; $n = 8$. *$P < 0.0001$, **$P < 0.0005$ vs. untreated cells. *B*: Seventy-two hours after transfection, CDM3D and cFLIP CDM3D cells were not treated or treated for 6 h with IL-1β (100 units/ml) or IL-1β and TNF-α (100 units/ml each). Exposure to cytokines induced an increase in NF-κB transcriptional activity, and the effect was higher in cFLIP-expressing cells. Data are means ± SE of two independent experiments, each performed in triplicate. *$P < 0.05$ vs. IL-1β–treated CDM3D cells; **$P < 0.005$ vs. IL-1β/TNF-α–treated CDM3D cells.**

Switzerland), 2.5% fetal bovine serum (Life Technologies), 10 mmol/l HEPES, 1 mmol/l Na-pyruvate, and 2 mmol/l glutamine, at 37°C with 5% $CO_2$.

**Preparation of lentiviral vectors and infection of CDM3D cells.** The human cFLIP cDNA, provided by Dr. J. Tschopp (Department of Biochemistry, University of Lausanne) (29), contains an octapeptide tag (FLAG) epitope at the 5′ end. It was subcloned into a modified SIN-18/phosphoglycerate kinase/woodchuck hepatitis virus (SIN-18-PGK-WHV) vector (42,43), which contains a neomycin resistance gene downstream of an internal ribosome entry site from encephalomyocarditis virus, provided by Dr. N. Déglon (University Hospital, Lausanne, Switzerland). High-titer stocks of lentiviral vectors carrying the PGK-driven lacZ or cFLIP genes packaged by the multiply attenuated lentivirus CMVΔR8.91, and pseudotyped with the vesicular stomatitis virus-G envelope protein (plasmid pMD-G), were prepared by transient transfection of 293T cells as described (39,44,45). cFLIP and lacZ virus titers were determined by p24 enzyme-linked immunosorbent assay (ELISA) (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. CDM3D cells were transduced with a multiplicity of infection of 10–20. Selection of the pool of infected cells was initiated 48 h after infection by adding 800 μg/ml G418 (an analog of neomycin) for 1 week, followed by 400 μg/ml of the drug for an additional week. At the end of the selection period, all surviving and growing cells expressed the transgene in various amounts.

**Analysis of cFLIP expression by Northern and Western blot.** Total RNA was isolated and analyzed by Northern blot as described previously, using specific probes prepared by random-primer labeling (46). Densitometry scanning of the blots was performed using the Image FX phosphorimager (Bio-Rad). Cell lysates were immunoassayed as previously described (47), and the cFLIP protein level was determined in cell lysates prepared in 5% SDS, 5 mmol/l EDTA, and 80 mmol/l Tris (pH 6.8), with 50 μg/ml phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin. cFLIP protein was detected in immunoblot using the anti–Flag-M2 mouse monoclonal antibody (Sigma).

**Insulin secretion.** Cells were plated in 24-well dishes at a density of $10^5$ cells/well for 48 h before incubation with cytokines. After cytokine exposure for 48 h, cells were then incubated for 1 h in HEPES-buffered Krebs-Ringer bicarbonate buffer (KRBH), pH 7.4, containing 0.5% BSA with 2.8 mmol/l glucose and 200 μmol/l isobutylmethylxanthine (IBMX) (Sigma Chemie, Buchs, Switzerland). The medium was changed and cells were incubated again for 1 h in KRBH/0.5% BSA containing 2.8 or 16.7 mmol/l glucose and IBMX. Secreted insulin was quantitated by radioimmunoassay (Linco Research, Labodia, Yens, Switzerland) as described (39,48). Intracellular insulin was measured in acid-ethanol cell lysates. Briefly, cells were lysed in 250 μl of 75% ethanol and 1.5% concentrated hydrochloric acid. Aliquots of cell lysates were also analyzed for DNA content (49) to normalize the secretion data. Lysates in acid ethanol were neutralized with 1/10 volume of 1 mol/l $Na_2CO_3$, and DNA content was determined by fluorescence using a Fluoroskan-II microplate fluorometer (Labsystems, Helsinki, Finland) with excitation filter set at 355 nm and emission filter set at 460 nm.

**Protease activity.** The activation of the caspase-8 protease was determined using a commercially available kit (ApoAlert Caspase-8 colorimetric assay kit; Clontech Laboratories, Palo Alto, CA) according to the manufacturer's specifications. This method is based on spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) after cleavage from the protease substrate IETD-pNA, characteristic of the caspase-8 cleavage site. Briefly, crude lysates from cytokine-stimulated or untreated cells were recovered, and total proteins (100 μg) were incubated in the presence of 200 μmol/l IETD-pNA. After 2 h of incubation at 37°C, the absorbance at 405 nm was measured using an ELISA plate reader. Comparison of the colorimetric reaction from cytokine-treated samples with an uninduced control allowed quantification of the increase in protease activity.

**Transient transfection and luciferase assays.** Cells were seeded in 24-well dishes at a density of $10^5$ cells/well 48 h before transfection with the indicated plasmids using the LipofectAmine-2000 reagent (Roche Molecular Biochemicals, Rotkreuz, Switzerland). A total of 1 μg DNA was transfected, which consisted of 0.8 μg of a NF-κB-luciferase reporter plasmid (provided by Dr C. Widmann, Institute of Cellular Biology and Morphology, University of Lausanne, Switzerland) or 0.8 μg of an iNOS-luciferase reporter plasmid (piNOS-1002luc), containing 1,002 bp of the rat iNOS promoter linked to luciferase (provided by Dr. D. Eizirik, Diabetes Research Center, Vrij Universiteit

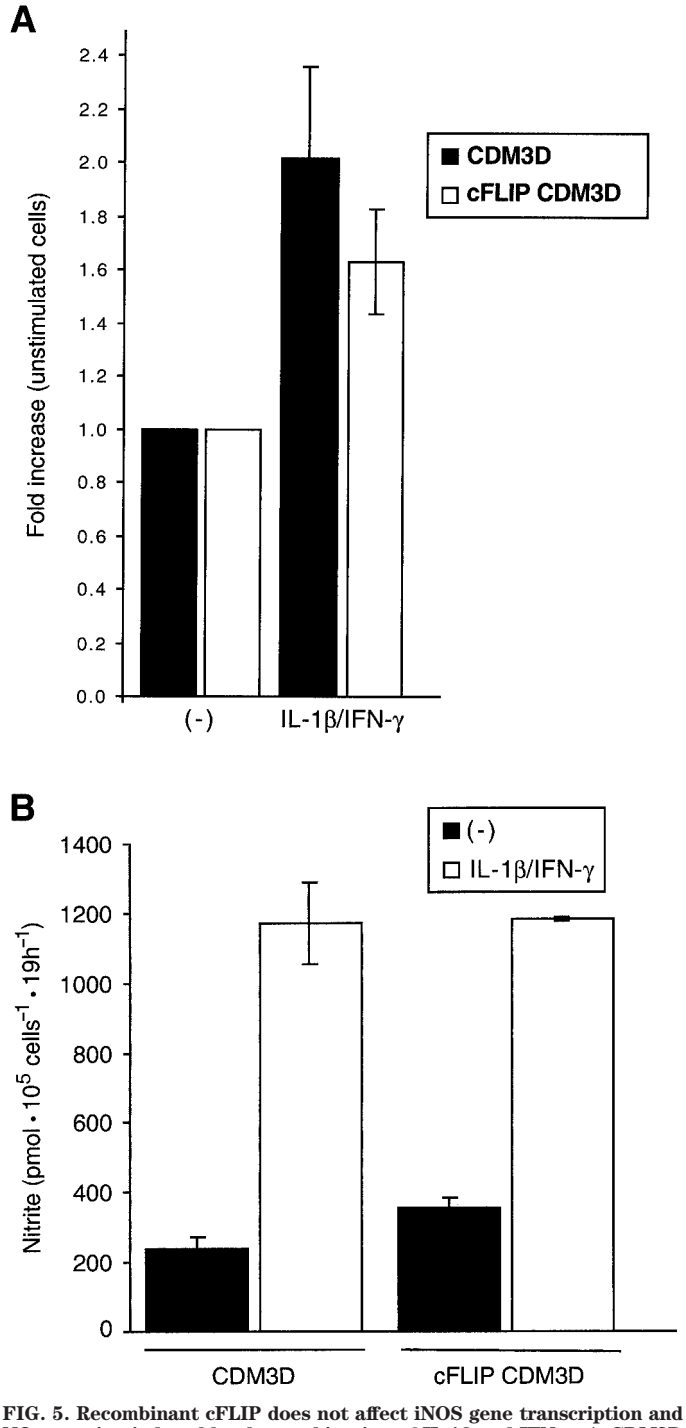



**FIG. 5. Recombinant cFLIP does not affect iNOS gene transcription and NO secretion induced by the combination of IL-1β and IFN-γ. *A*: CDM3D and cFLIP CDM3D cells were cotransfected with an iNOS-luciferase reporter as well as a control cytomegalovirus-lacZ construct to assess for transfection efficiency. Seventy-two hours after transfection, the cells were stimulated with 10 units/ml IL-1β plus 150 units/ml IFN-γ, and luciferase as well as β-galactosidase activities were measured 6 h after stimulation. Exposure to cytokines induced a similar increase in iNOS reporter activity in cFLIP-expressing cells and control CDM3D cells. Data are means ± SE of two independent experiments, each performed in triplicate. *B*: Nitrite accumulation was measured, and data were normalized to the protein content of the wells as previously described (41). CDM3D and cFLIP CDM3D cells were stimulated for 19 h with the same concentration of cytokines as in *A*. Upon cytokine exposure, cFLIP-expressing cells showed a similar increase in NO secretion compared with CDM3D cells. Data are means ± SE; $n = 3$.**

Brussel, Brussels, Belgium), and 0.2 μg of a β-galactosidase reporter plasmid (driven by the cytomegalovirus promoter) that was used to correct for transfection efficiency. Seventy-two hours after transfection, cells were stimulated with cytokines for determined times, and relative activity of luciferase and β-galactosidase was determined as described (40).

**Apoptosis.** Two days before induction of apoptosis, cells were seeded in a polylysine-treated 96-well microtiter plate ($10^4$ cells/well). The medium was changed and cells were treated or not for 36 h with a combination of TNF-α, IL-1β, and IFN-γ ($10^3$ units/ml each). The percentages of viable and apoptotic cells were assessed as described previously (39). Medium was removed from the wells and replaced with the same volume of medium containing 20 μg/ml Hoechst 33342 (Fluka, Buchs, Switzerland) and 10 μg/ml propidium iodide (Sigma). After 5 min at room temperature, the cells were examined with an inverted fluorescence microscope with ultraviolet excitation at 340–380 nm. In each experimental condition, at least 500 cells were counted. A control plate was analyzed in parallel to determine spontaneous cell death, which was deduced from the experimental values.

## RESULTS

**Human cellular FLIP is stably expressed in CDM3D cells.** To evaluate whether it was possible to interfere with the TNF-α apoptotic response, we transferred the cFLIP cDNA in βTc-Tet and CDM3D cells using a recombinant lentivirus. Pools of transduced cells were selected in the presence of G418 and cFLIP transcripts, and proteins were detected by Northern (Fig. 1*A*) and Western (Fig. 1*B*) blot analysis, respectively. In transiently transfected 293T cells, the full-length 55-kDa form of cFLIP ($cFLIP_L$), as well as a COOH-terminally truncated form of the recombinant protein (43 kDa), were detected. In transduced CDM3D cells, only the full-length cFLIP was observed. We further assessed whether these cells were still able to secrete insulin in response to glucose. At high glucose concentrations, a three- to sevenfold stimulation of insulin secretion was observed (data not shown; see also Fig. 6).

**Cytokine-induced activation of caspase-8 is suppressed in the presence of cFLIP.** To investigate whether cytokine-induced apoptosis in mouse βTc-Tet cells was correlated with caspase activation, and to assess the role of cFLIP in the TNF-α–induced apoptotic pathway, the level of caspase-8 activity was evaluated in CDM3D and cFLIP CDM3D cells exposed for 5 h to TNF-α ($10^3$ units/ml) and the protein synthesis inhibitor cycloheximide (CHX) (0.5 μg/ml) or to a combination of $10^3$ units/ml of TNF-α, IL-1β, and IFN-γ. Figure 2 shows that in control and lacZ-expressing CDM3D cells, a ~1.6- to 2-fold increase in the activity of caspase-8 was observed upon treatment with TNF-α/CHX or the three cytokines. In contrast, in cFLIP-expressing cells, cytokine-induced activation of caspase-8 was completely abolished.

**Expression of cFLIP protects CDM3D cells against cytokine-induced apoptosis.** Whereas cytokine-induced apoptosis of βTc-Tet cells was partially prevented by expression of Bcl-2, the additional presence of mutant proteins blocking the IL-1β signaling pathway conferred an increased resistance to the induction of cell death (39,40). To address the question of whether interfering with TNF-α signal transduction would also provide an increased protection against cytokine-induced apoptosis, cells were exposed for 36 h to a combination of IL-1β, TNF-α, and IFN-γ ($10^3$ units/ml each). As shown in Fig. 3, whereas viability of CDM3D cells was reduced by 60–70%, viability of cFLIP CDM3D cells was reduced by only ~35%.

**The transcriptional activity of NF-κB is increased in βTc-Tet and CDM3D cells expressing cFLIP.** In addition to the protective effect of cFLIP on cytokine-induced caspase-8 activation and apoptosis, we addressed the question of whether cFLIP per se was sufficient to modify

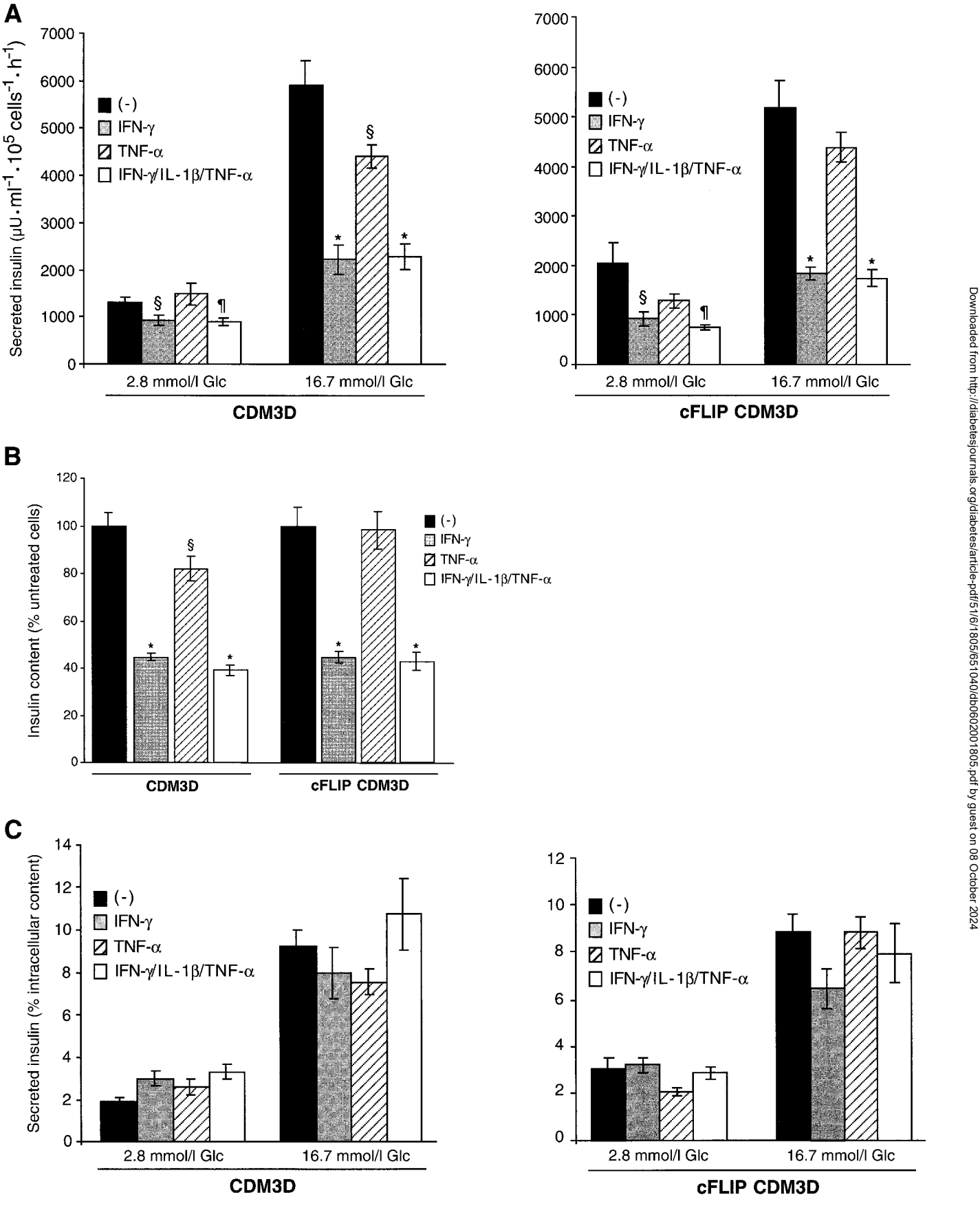



**FIG. 6. cFLIP and cytokine-mediated reduction of glucose-stimulated insulin secretion. Cells were not treated or treated for 48 h with IFN-γ (150 units/ml), TNF-α ($10^3$ units/ml), or a combination of IFN-γ plus IL-1β (10 units/ml) and TNF-α (100 units/ml). Insulin secretion was then evaluated after 1-h incubation in the presence of 2.8 or 16.7 mmol/l glucose. *A*: At low and high glucose concentrations in CDM3D cells, insulin secretion was markedly**

the basal transcriptional activity of NF-κB. We therefore evaluated whether the stable expression of cFLIP in βTc-Tet and CDM3D cells would result in an increase in the transcriptional activity of NF-κB. Cells were transiently transfected with a NF-κB-luciferase reporter gene, and luciferase assays were performed 72 h posttransfection. As shown in Fig. 4*A*, NF-κB–driven luciferase activity was increased by 10-fold in βTc-Tet cells expressing cFLIP and by 2-fold in cFLIP CDM3D cells compared with the respective parental cell lines. We also observed that the basal NF-κB–dependent transcriptional activity was enhanced in CDM3D cells stably expressing Bcl-2 compared with βTc-Tet cells, as reflected by a higher NF-κB–driven luciferase activity. Furthermore, upon 6-h exposure to IL-1β (100 units/ml) and IL-1β plus TNF-α (100 units/ml each), cFLIP CDM3D cells displayed a higher stimulation of NF-κB transcriptional activity compared with cytokine-treated CDM3D cells (Fig. 4*B*).

**Recombinant cFLIP does not affect iNOS gene transcription and NO secretion in response to IL-1β and IFN-γ.** We have shown previously (40) that the upregulation of iNOS gene and NO production in our cellular model was dependent on the presence of both IL-1β and IFN-γ, as has also been described in human and mouse primary β-cells, and no additional effect of TNF-α was observed. In addition, we also reported that iNOS expression and nitrite production were completely suppressed by blocking either the IL-1β or IFN-γ intracellular signaling pathway (40,41). To further address the question whether the stable expression of cFLIP may affect the ability of CDM3D cells to induce iNOS expression and NO secretion, the cells were transiently transfected with an iNOS-luciferase reporter gene. The cells were then treated for 6 h with IL-1β (10 units/ml) and IFN-γ (150 units/ml) before measuring luciferase activity. As shown in Fig. 5*A*, the activation of the iNOS reporter construct (~1.7-fold) in response to cytokines was similar in cFLIP-expressing cells and CDM3D cells. In agreement with the above results, 19-h exposure to IL-1β combined with IFN-γ resulted in an increase in nitrite production that did not differ between cells expressing cFLIP or not (Fig. 5*B*).

**cFLIP and cytokine-induced reduction of glucose-stimulated insulin secretion.** To determine whether expression of cFLIP would protect cytokine-treated cells against impaired GSIS, secretion experiments were performed with cells exposed to cytokines. Cells were first treated for 48 h with IFN-γ (150 units/ml), TNF-α ($10^3$ units/ml), or a combination of IL-1β (10 units/ml) plus TNF-α (100 units/ml) and IFN-γ (150 units/ml) and then incubated for 1 h at 2.8 mmol/l glucose before being exposed to 2.8 or 16.7 mmol/l glucose for 1 h. Figure 6*A* shows that insulin secretion, at low and high glucose concentrations, was markedly reduced in CDM3D cells after exposure to IFN-γ alone or the three cytokines and that cFLIP did not prevent this inhibitory effect. At 16.7 mmol/l glucose, exposure of CDM3D cells to a high concentration of TNF-α resulted in a 25 ± 4% decrease in GSIS, whereas no significant reduction was observed in cFLIP-expressing cells. As shown in Fig. 6*B*, impaired secretion in CDM3D and cFLIP CDM3D cells was correlated with a reduction of intracellular insulin levels, which reached 50–60% following treatment with IFN-γ or the combined cytokines. Similarly, insulin content was decreased by 18 ± 5% in CDM3D cells after exposure to high amounts of TNF-α, and the presence of cFLIP prevented this reduction (Fig. 6*B*). When secretion was expressed as a percentage of the total intracellular insulin content, the secretion rate was not affected in cytokine-treated cells compared with untreated cells (Fig. 6*C*).

## DISCUSSION

In this study, we report that exposure of CDM3D cells to TNF-α, in the presence of CHX or IL-1β and IFN-γ, leads to the activation of caspase-8. We show that lentivirus-mediated stable expression of cFLIP in CDM3D cells suppressed TNF-α–induced caspase-8 activation and protected the cells against cytokine-induced apoptosis. Furthermore, cFLIP overexpression is associated with an increase in the basal and cytokine-induced transcriptional activity of NF-κB, whereas it does not affect IL-1β/IFN-γ–mediated iNOS gene upregulation and NO secretion. Finally, the reduction in GSIS induced by TNF-α was prevented by cFLIP but not that induced by the combined action of TNF-α, IFN-γ, and IL-1β, indicating that other cytokine-activated intracellular signaling pathways induce the secretory dysfunction.

We previously observed that the induction of apoptosis in CDM3D cells required the combined action of TNF-α, IFN-γ, and IL-1β (39,40). Here we show that cytokine-induced apoptosis is correlated with the activation of caspase-8, an initiator caspase participating in the TNF-α apoptotic cascade. The major role of caspases in inducing apoptosis is well established (50), and their role in cytokine-dependent apoptosis in β-cells has also been suggested by a number of studies (6,51). For instance, in human and mouse islets, as well as in β-cell lines, IFN-γ on its own induces the upregulation of IL-1–converting enzyme (ICE/caspase-1), and caspase-1 (52) is able to process and activate the effector caspase-3.

TNF-α–dependent activation of caspase-8 in CDM3D cells requires the inhibition of protein synthesis, indicating that short-lived proteins may protect against apoptosis. Alternatively, caspase-8 activation and apoptosis of CDM3D cells can be induced by the action of TNF-α associated with IFN-γ and IL-1β. These results therefore suggest that these two cytokines may participate in the repression of anti-apoptotic proteins or, conversely, in the induction of pro-apoptotic molecules, or both. We recently demonstrated that cytokine-induced apoptosis in mouse βTc-Tet cells could also be suppressed by inactivating the IFN-γ–dependent activation of the Janus kinase-signal transducer and activation of transcription (JAK/STAT)

**reduced by IFN-γ or by the combination of cytokines, and high amounts of TNF-α induced a slight reduction of GSIS at high glucose concentrations. cFLIP protected cells against TNF-α inhibitory action but not against that induced by IFN-γ or the three cytokines. Significant differences versus untreated cells: *$P < 0.0001$, §$P < 0.05$, ¶$P < 0.01$. *B*: Intracellular insulin content was strongly decreased (50–60%) by IFN-γ or by the three cytokines, and was reduced by ~18% by TNF-α. Whereas cFLIP protected the cells against TNF-α action, no protection against IFN-γ and the three cytokines could be observed. *$P < 0.0001$, §$P < 0.05$ vs. untreated cells. *C*: Secretion of insulin at 2.8 and 16.7 mmol/l glucose, expressed relative to insulin content, was not significantly modified following treatment with cytokines. Data are means ± SE of two independent experiments, each done in triplicate.**

pathway by overexpressing the SOCS-1 signaling inhibitor (41). The signaling pathways activated by TNF-α and IFN-γ and leading to apoptosis are complex and highly interactive. Several reports have described that STAT-1–mediated induction of apoptosis in response to IFN-γ was dependent on the expression of caspases such as caspase-1, -3, and -8 (51,53,54). IFN-γ may also prime the cells to respond to the cytotoxic action of TNF-α in part by inducing caspase-8 but also, possibly, by upregulating STAT-1 and IFN regulatory factor (IRF)-1 (55). On the other hand, TNF-α was shown to activate the JAK/STAT pathway by increasing both JAK and STAT-1 tyrosine phosphorylation (56,57). A role of TNF-α in controlling the JAK/STAT pathway was also supported by data (51) showing that STAT-1–null cells were resistant to apoptosis induced by TNF-α and that reintroduction of STAT-1 restored both TNF-α–induced apoptosis and expression of caspases. A further interaction between the two cytokines' signaling pathways (58) is through binding of STAT-1 to the TNFR1-TRADD signaling complex, which favors the formation of the DISC required for induction of apoptosis.

If cFLIP acts as an inhibitor of caspase-8 activation, its stable overexpression in CDM3D cells should block TNF-α–induced caspase-8 activation and apoptosis. We indeed demonstrated that the presence of cFLIP prevented cytokine-induced activation of caspase-8 and was correlated with increased resistance to apoptosis. Activation of the NF-κB and apoptotic pathways downstream of the TNF-α receptor depend on FADD and TRADD binding to the receptor death domain. cFLIP interaction with the DISC may therefore favor activation of the other pathway, leading to increased NF-κB activity, and act as an important determinant of cell survival/apoptosis balance. Indeed, we observed that stable expression of cFLIP in βTc-Tet and CDM3D cells resulted in a marked increase (2- to 10-fold) in NF-κB transcriptional activity. Therefore, our results indicate that overexpressed cFLIP may act not only by competing with caspase-8 recruitment and activation at the DISC, but also by triggering the activity of NF-κB. On the other hand, whether cFLIP-dependent activation of NF-κB leads to upregulation of anti- or pro-apoptotic genes is not known. Indeed, NF-κB may have opposite effects on apoptosis in a single cell type, depending on the stimulus (59). However, there is evidence showing that NF-κB participates in pro-apoptotic events in β-cells (60,61). Furthermore, we also showed that the presence of cFLIP correlated with increased IL-1β–mediated transcriptional activity of NF-κB. This is in contrast, however, to the observation that cFLIP did not enhance cytokine-induced iNOS gene transcription and nitrite production. Thus, these results seem to indicate that the fraction of IL-1β–induced NF-κB activity dependent on cFLIP does not have an impact on the regulation of iNOS and NO in CDM3D cells in response to cytokines.

Exposing CDM3D cells to cytokines strongly reduces GSIS and intracellular insulin content (~60%). We previously observed that this effect could be explained by the sole action of IFN-γ, which could be completely prevented by blocking the JAK/STAT pathway by overexpression of SOCS-1 (41). Upon exposure to high concentrations of TNF-α, a weak inhibitory effect (~20%) on cellular insulin content and secretion was nevertheless observed, which was suppressed in the presence of cFLIP. In primary mouse islets, exposure to TNF-α leads to a 40–50% reduction in intracellular insulin level (7,62). TNF-α may therefore also negatively affect insulin response in mouse β-cells. Although the mechanism by which this occurs is not known, it may possibly involve activation of the JAK/STAT pathway, as described above.

Transplantation of encapsulated, insulin-secreting β-cells may provide the most efficient therapy for type 1 diabetes. In this therapeutic approach, however, the transplanted cells have to face several challenges. First, they are exposed to unfavorable oxygen and nutrient gradients due to high cell density and the presence of an immunoisolation barrier. Second, they need to withstand the action of cytokines released by inflammatory and reactivated immune cells. We previously showed that transfer of the Bcl-2 gene into βTc-Tet (CDM3D) cells conferred protection against stress- and hypoxia-induced apoptosis (39). Stable expression of dominant-negative mutants of MyD88 (40) and overexpression of SOCS-1 (41) blocked the IL-1β and IFN-γ intracellular signaling pathways, respectively, and both increased the resistance to cytokine-induced apoptosis. In addition, blocking the IFN-γ pathway with SOCS-1 prevented decreased cellular insulin content and impaired GSIS. Here we demonstrate that cFLIP abolishes cytokine-induced caspase-8 activation and apoptosis and, in addition, might favor activation of the protective NF-κB pathway.

Together, our data indicate that cytokines can activate distinct pathways to induce β-cell dysfunction and apoptosis. On the one hand, IFN-γ action is sufficient to markedly reduce insulin gene expression and GSIS. On the other hand, cell death requires the combined presence of TNF-α, IL-1β, and IFN-γ, even though blocking either intracellular signaling pathway is sufficient to increase resistance to apoptosis, indicating that these three pathways must converge to induce apoptosis. Transplantation of cells with inhibition of selected cytokine intracellular signaling pathways in autoimmune diabetic mice may allow us to determine the contribution of each pathway in graft rejection. Furthermore, these studies may allow us to evaluate whether genetic modifications, alone or in combination with cellular encapsulation, can permit cell survival and function. These studies will be important in determining cellular parameters required for successful cell transplantation therapy of type 1 diabetes.

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