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Mechanisms of T-cell protection from death by IRX-2: a new immunotherapeutic

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Abstract

Objectives—IRX-2 is a novel immunotherapeutic containing physiologic quantities of several cytokines which protects human T lymphocytes from tumor-induced or drug-induced apoptosis. Here, we investigate the mechanisms responsible for IRX-2-mediated protection of T lymphocytes exposed to tumor-derived microvesicles (TMV).

Methods—Jurkat cells or primary human T cells ± IRX-2 were co-incubated with TMV and then examined by flow cytometry or Western blots for expression of molecules regulating cell survival (FLIP, Bcl-2, Bcl-xL, Mcl-1) or death (Fas, caspase 8, caspase 9, Bax, Bid). ANX V binding, caspase activation or cytochrome c release were also measured ± cycloheximide (CHX) or ± the Akt-specific inhibitor. Jurkat cells transfected with the cFLIP gene were used to evaluate the role of cFLIP in IRX-2-mediated protection. Effects of CHX on IRX-2-mediated protection and activation of NF-κB upon the TMV/IRX-2 treatment were also measured.

Results—IRX-2 protected T cells from apoptosis by preventing Fas overexpression induced by TMV and blocking caspase 8 activation by up-regulating cFLIP. Jurkat cells overexpressing

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cFLIP were more resistant to TMV-induced apoptosis than the mock-transfected cells ($p < 0.02$). Signaling via the PI3K/Akt pathway, IRX-2 corrected the imbalance of pro- versus anti-apoptotic proteins induced by TMV and promoted NF-κB translocation to the nucleus. CHX abolished IRX-2-mediated protection in T cells, suggesting that IRX-2 induces de novo synthesis of one or more proteins that are required for protection.

Conclusions—This biologic may be therapeutically useful for protection of activated T cells from tumor-induced immune suppression and death.

Keywords

IRX-2; Tumor-derived exosomes; Apoptosis; Lymphocyte survival; cFLIP; PI3K/Akt; Bcl-2 proteins

Introduction

The role of the adaptive immune system in generating protective responses to developing tumors has been established in recent years [1]. Tumor-rejection antigens have been identified for a variety of human malignancies [2], and circulating $CD8^+$ cytotoxic T lymphocytes (CTL) able to recognize peptides derived from these antigens are detectable in the blood of patients with cancer [3–5]. The induction and expansion of tumor-specific CTL is a major goal of currently available cancer vaccines [6]. However, the effectiveness of CTL generated by anti-tumor vaccines in cancer patients is often compromised by tumor escape mechanisms, including death of tumor-antigen-specific effector T cells [7, 8]. This targeted death of activated T cells, especially of $CD8^+$ T cells, within the tumor microenvironment as well as the peripheral circulation of cancer patients, could be mediated by tumor-derived microvesicles (TMV) [9, 10]. The presence of such FasL-bearing TMV in the sera of cancer patients correlates with apoptosis and TCR alterations in $CD8^+$ effector T cells and with an increased tumor burden and nodal involvement in these patients [11–14]. Utilizing the Fas/FasL killing mechanism and the release of membrane-bound FasL through secretion of TMV, tumors effectively eliminate $CD8^+$ cytotoxic T cells which are necessary for anti-tumor host defense. The loss of these cells could explain the inadequate immune responses to cancer vaccines, underscoring the need for the development of strategies for protection of activated T cells from tumor-induced death.

In a previous study we have shown that IRX-2, a novel immunotherapeutic agent containing physiological quantities of several cytokines, protects activated T lymphocytes from apoptosis mediated by TMV [15]. This protection involves the inhibition of TMV-mediated activation of caspase 8 in the receptor-mediated pathway as well as the protection of the mitochondrial membrane from disruption, cytochrome c release and activation of membrane-associated apoptogenic proteins. Moreover, the cytoprotective effects of IRX-2 associated with the re-expression of the TCR-associated CD3ζ-chain, Jak-3 and Stat-5, were mediated by the PI3K/Akt survival pathway [15].

In this study, we further define the IRX-2-mediated mechanisms protecting human T lymphocytes from TMV-mediated apoptosis. Changes in the expression of anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, FLIP) and pro-apoptotic (Bax, Bim, Fas) proteins after treatment with TMV ± IRX-2 are correlated with sensitivity/resistance of T cells to apoptosis. Using specific inhibitors or cycloheximide, we demonstrate the involvement of Akt and a newly synthesized protein, respectively, in IRX-2-mediated protection of lymphocytes from TMV-mediated apoptosis.

Materials and methods

Antibodies and reagents

The following monoclonal antibodies (mAbs) were used for flow cytometry analysis: anti-CD3-ECD, anti-CD8-PC5, anti-CD4-PE (Beckman Coulter); anti-Bcl-2-FITC, anti-Bcl-2-PE, anti-Fas-FITC, anti-FasL-PE (BD Biosciences, San Jose, CA); anti-Bax-FITC, anti-Bcl-xL-FITC (Santa Cruz Biotechnology) and anti-Bid-mAb (Abcam Inc). Polyclonal antibodies (Abs) were: anti-Bim (Cell Signaling), anti-FLIP (GenWay Biotech), and anti-Mcl-1 (Santa Cruz Biotechnology). FITC-conjugated annexin V was purchased from Beckman Coulter. FITC-conjugated anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories, and the isotype controls (IgG_1-FITC, IgG_{2a}-FITC and IgG_{2b}-FITC and IgG2-PE) were purchased from BD Biosciences. Antibodies used for Western blot analysis included: polyclonal anti-human caspase 8, monoclonal anti-human caspase 9 (BD Pharmingen) and polyclonal anti-FLIP (GenWay Biotech). Anti-Fas (CH-11) agonistic mAb, IgM isotype control for CH-11, anti-Fas blocking mAb, clone ZB4, and isotype IgG1 control for ZB4 were all purchased from Upstate Biotechnology. All cell culture reagents including AIM V and RPMI 1640 media, phosphate-buffered saline (PBS), heat-inactivated fetal calf serum (FCS), streptomycin, penicillin, L-glutamine, recombinant trypsin-like enzyme (TrypLE) and the trypan blue dye were purchased from Gibco/Invitrogen. Bovine serum albumin (BSA), saponin, and cyclohexemide were from Sigma-Aldrich. 7-amino-actinomycin D (7-AAD) and the pan caspase inhibitor, z-VAD-FMK, were obtained from BD Biosciences. The selective inhibitor of Akt1/Akt2 was purchased from Calbiochem and the selective inhibitors for caspase 3, caspase 8 and caspase 9 from R&D Systems.

IRX-2 description

IRX-2 is a primary lymphoid cell-derived biologic provided by IRX Therapeutics, which is currently in clinical trials for treatment of cancer. IRX-2 contains nanogram quantities of IL-1β, IL-2, IL-6, IL-8, GM-CSF, interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α). IRX-2 is prepared under cGMP standards from phytohemagglutinin (PHA)-stimulated normal human peripheral blood mononuclear cells (PBMC) as previously described [15]. Quality control tests including a bioassay with CTLL cells and quantitative enzyme-linked immunosorbent assays (ELISA) for cytokine levels are routinely performed to guarantee the consistency of consecutive IRX-2 preparations. For all experiments reported here, a 1:3 dilution of IRX-2 was used which contains the following cytokine concentrations: IL-1β: 0.3; IL-2: 4.3; IL-6: 0.7; IL-8: 25.2; GM-CSF: 0.6 IFNγ: 2.2; TNFα: 1.6 (all in ng/mL).

Cells and cell lines

The HNSCC cell line, PC-13, was established and maintained in our laboratory and was retrovirally transfected with the human FasL gene obtained from Dr. S. Nagata (Osaka Biosciences Institute) as previously reported [16]. Supernatants of transfected PCI-13-cells (PCI-13-FasL), which contained both sFasL and the 42 kDa membrane form of FasL, were used as a source of TMV. Jurkat cells were obtained from American Tissue Culture Collection (ATCC) and were transfected with CD8 cDNA by Dr. H. Rabinowich, University of Pittsburgh. The $CD8^+$ Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, L-glutamine and antibiotics. T lymphocytes were isolated from peripheral blood mononuclear cells (PBMC) obtained from consented normal donors (IRB#980633). PBMC were isolated on Ficoll-Hypaque density gradients (GE Healthcare Bio-Sciences Corp), washed and plated in T162 culture flasks in an atmosphere of 5% CO_2 for 1 h at 37°C to remove $CD14^+$ monocytes. The non-adherent T-lymphocyte fraction was immediately used for experiments or cryopreserved. $CD8^+$ T cells or $CD4^+$ T cells were purified by positive selection using AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. Purified $CD8^+$ or $CD4^+$ T cells were cultured for 2–3 days in

AIM V medium supplemented with 10% FBS (previously depleted of MV by ultracentrifugation) in the presence of beads coated with anti-CD3 and anti-CD28 antibodies (T Cell Activation/Expansion Kit, Miltenyi Biotec). All cells used for the above described experiments were in the log phase of growth.

Isolation of microvesicles

TMV were isolated from culture supernatants of the FasL-transfected PCI-13 cell line as described [13]. Briefly, the concentrated cell culture supernatants were fractioned by a two-step procedure, including size exclusion chromatography on a Sepharose 2B column and ultracentrifugation at 105,000×*g* for 2 h at 4°C. The protein concentration in each TMV preparation was estimated by a Lowry's protein assay (Bio-Rad Laboratories).

Western blot assays

To determine caspase 8 and caspase 9 activation in Jurkat cells or Jurkat cells transfected with cFLIP, the cells were co-incubated with TMV ± IRX-2 at 37°C for various periods of time. Cells treated with anti-Fas agonistic mAb, CH-11, for 4 h served as positive controls. The cells were then washed, centrifuged at 4°C and lysed in equal volumes of ice-cold lysis-buffer (50 mM Tris–HCL pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40) and a protease inhibitor cocktail (Pierce Chemical Co). Equivalent protein quantities, as determined by Lowry, were loaded on each gel. The proteins were separated by SDS-PAGE and electrotransfered to polyvinylidene difluoride (PVDF) membranes which were blocked, incubated overnight at 4°C with the appropriate antibodies, washed and developed as previously described [15].

Co-incubation of Jurkat cells or activated normal T-lymphocytes with TMV and IRX-2

Jurkat cells or activated primary T lymphocytes were plated at 0.3×10^6 cells per well in a 96-well plate and pre-treated or not with IRX-2. TMV (10 μg protein/0.3×10^6 cells) were then added for 4–24 h. In some experiments, 0.1 μg/mL cycloheximide (CHX) was added alone or in combination with IRX-2 for 24 h prior to TMV. In selected blocking experiments, anti-Fas neutralizing monoclonal antibody, ZB-4, the pan-caspase inhibitor, Z-VAD-FMK, or the specific Akt-inhibitor or specific inhibitors for caspase 3, 8 and 9 were added at the indicated concentrations prior to TMV.

Cell surface staining

Jurkat cells or activated primary T-lymphocytes (at least 300,000 cells/tube co-incubated with TMV and/or IRX-2) were washed twice in buffer (0.1% w/v BSA and 0.1% w/v NaN_3) and stained for cell surface markers as described [15]. Briefly, cells incubated with the optimal dilution of each Ab for 20 min at RT in the dark were washed twice with buffer and fixed in 1% (v/v) paraformaldehyde in PBS. The following Abs were used for surface staining: anti-CD3-ECD, anti-CD4-PE, anti-CD8-PC5, anti-Fas-FITC and anti-FasL-PE. The appropriate isotype control Abs were used in all experiments.

Flow cytometry

Four color flow cytometry was performed using a FACScan flow cytometer (Beckman Coulter) equipped with Expo32 software (Beckman Coulter). Lymphocytes were gated based on FS and SS and at least 10^5 cells were collected for analyses. Gates were restricted to the $CD3^+CD8^+$ or $CD3^+CD4^+$ T-cell subsets for the analysis of activated primary T lymphocytes. Data were analyzed using Coulter EXPO 32vl.2 analysis software.

Annexin V binding assay

Annexin V (ANX) binding to TMV and/or IRX-2 co-incubated $CD8^+$ Jurkat cells or activated T lymphocytes was measured by flow cytometry to evaluate spontaneous or in vitro induced apoptosis as described [15].

Measurements of caspase activation

Pan-caspase activity was tested by intracellular staining for activated caspases using a pan-caspase inhibitor, CaspACE™ FITC-VAD-FMK (Promega). Cells were resuspended in PBS and FITC-VAD-FMK was added at a final concentration of 5 μM. The cells were incubated for 20 min at 37°C washed with PBS, stained for cell surface markers, fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Evaluation of apoptosis-related proteins

Expression of anti-apoptotic proteins Bcl-2, Bcl-xL, cFLIP and Mcl-1 and the pro-apoptotic proteins Bax, Bim and Bid was investigated in Jurkat cells or activated primary T lymphocytes by flow cytometry. The cells were stained for surface T-cell markers as described above and were then fixed with 1% paraformaldehyde in PBS at RT for 10 min. They were permeabilized with saponin (0.1% v/v in PBS) for 15 min at 4°C. Next, the cells were stained for 30 min at 4°C with FITC- or PE-conjugated anti-human Bcl-2, Bax and Bcl-xL or unconjugated Abs specific for cFLIP, Bim, Bid or Mcl-1, followed by washing with 0.1% saponin. Samples stained with unconjugated Abs were further incubated with an FITC-conjugated goat anti-rabbit IgG for 15 min at RT. After washing with 0.1% saponin, cells were fixed in 1% paraformaldehyde. Isotype control Abs were used for surface and intracellular staining, and all Abs were pre-titered using fresh PBMC.

Activation of NF-κB

To measure NF-κB activation, Jurkat cells were co-incubated in 96-well plates with IRX-2 or with TMV or with IRX-2 + TMV for 2 h. TNF-α was used as positive control. The cells were then stained with an Ab specific for the p65 subunit of NF-κB. Briefly, cells were centrifuged, fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.2% Triton-X for 1 h and stained for p65 using polyclonal rabbit anti-human p65 Ab (2 μg/mL; Santa Cruz Biotechnology). The cells were washed in 1% (w/v) BSA in PBS and stained with donkey anti-rabbit FITC-labeled secondary Ab (1:200; Santa Cruz Biotechnology) for 1 h in the dark. Hoechst 33342 (Invitrogen) was used to stain cell nuclei. In negative controls, the primary Ab was omitted. Translocation of p65 into nuclei was measured using an ArrayScan microscope, and the results were calculated as mean fluorescence intensity (MFI) of p65 subunit translocated into nuclei.

Statistical analysis

Statistical analysis was performed using the Student's *t* test. p values < 0.05 were considered significant.

Results

IRX-2 normalizes the TMV-induced imbalance of pro- and anti-apoptotic proteins

To determine whether TMV-induced death of lymphocytes involved caspase activation, we co-incubated Jurkat cells with TMV in the presence of either a pan-caspase inhibitor or specific inhibitors of caspases 8, 3 or 9 (Fig. 1a). The pan-caspase inhibitor and the caspase 3 inhibitor reduced apoptosis of Jurkat cells by 50–70%. The inhibition of caspase 8 or caspase 9 was less effective in preventing TMV-induced apoptosis, as it reduced death of Jurkat cells by only 30%. Thus, TMV-induced apoptosis of T cells was only partially

caspase-dependent and involved caspases of the extrinsic and intrinsic apoptotic pathways. However, TMV also induced caspase-independent apoptosis events in T lymphocytes, since the inhibition of caspase activation did not completely abrogate TMV-induced cell death.

We have previously reported that TMV-treatment activates the mitochondrial apoptotic pathway, while co-incubation of T cells with IRX-2 prior to TMV treatment prevents this activation [15]. However, it was possible that IRX-2 alone had an impact on the expression of Bcl-2 proteins in T cells. In fact, IRX-2 increased the percentage of positive cells expressing the anti-apoptotic proteins FLIP, Bcl-2 and Mcl-1 (Fig. 1b, light gray bars) and up-regulated their expression level in Jurkat cells (Fig. 1c). TMV treatment of Jurkat cells caused a significant down-regulation in expression of the anti-apoptotic proteins Bcl-2, Bcl-xL, FLIP and Mcl-1, and this down-regulation was in part prevented by pre-treatment with IRX-2 (Fig. 1b, c). TMV also caused a significant increase in expression of the pro-apoptotic proteins Bax and Bim, and IRX-2 blocked this up-regulation. Similar results were obtained after IRX-2 and TMV were added to activated primary $CD8^+$ or $CD4^+$ cells (data not shown).

We next asked whether the modulation of Bcl-2 proteins by TMV and IRX-2 is caspase dependent. Thus, expression levels of Bcl-2, Bax, FLIP and Mcl-1 were measured by flow cytometry. Jurkat cells were co-incubated with TMV and IRX-2 in the presence of either the pan-caspase inhibitor or inhibitors of caspase 8 or 9. The pan-caspase inhibitor and, to a lesser extent, the caspase 8 inhibitor reduced the TMV-induced loss of Bcl-2 and FLIP, whereas inhibition of caspase 9 had a smaller but still significant effect (Supplementary Table 1). In contrast, the pan-caspase and caspase 8 inhibitors had no effect on Bax expression, whereas inhibition of caspase 9 significantly diminished the up-regulation of this pro-apoptotic protein by TMV. Blocking of caspase 9 activation had no or only minimal effects on the TMV-induced Mcl-1 modulation (Supplementary Table 1), while caspase 8 and pan-caspase inhibitors restored expression of Mcl-1. The inhibition of caspases had no effect on expression of pro- and anti-apoptotic proteins in the presence of IRX-2 alone (data not shown). Thus, while modulation of the Bcl-2 family members by TMV seems to require the involvement of specific caspases, the effect of IRX-2 alone on the expression of these proteins is caspase independent.

IRX-2-induced modulation of survival proteins is mediated by Akt

In previous experiments we showed that time-dependent Akt dephosphorylation induced by TMV was completely abolished by IRX pre-treatment [15]. To further define how IRX-2 engages the PI3K/Akt pathway to exert anti-apoptotic effects, we blocked Akt signaling using a specific Akt-1/-2 inhibitor. This completely abrogated the protective effect of IRX-2 in Jurkat cells (Fig. 2a). The inhibitor alone, used at the concentration of 5 μM, did not affect cell viability. In the presence of the inhibitor, IRX-2 lost its ability to prevent the TMV-induced activation of caspases 8 and 9 (Fig. 2b, two upper panels). Activated Akt was required not only for the IRX-2-mediated protection against Fas-mediated apoptosis but also for the protection against mitochondrial apoptotic pathways. In the presence of the Akt inhibitor, IRX-2 could not prevent the TMV-induced cytochrome c release into the cytosol (Fig. 2b, lower panel).

Akt is known to regulate cell survival by targeting the pro- and anti-apoptotic Bcl-2 family members [17]. We therefore asked whether Akt signaling was required for the IRX-2-mediated changes in expression levels of FLIP, Bcl-2, Bcl-xL, Mcl-1, Bax and Bim. While pre-treatment with IRX-2 increased the expression of anti-apoptotic FLIP, Bcl-2 and Mcl-1 in Jurkat cells (Fig. 2c), in the presence of the Akt inhibitor abrogated IRX-2-mediated up-regulation of FLIP and Bcl-2 expression. Akt inhibition did not influence the effect of MV on the expression levels of these proteins (data not shown). However, in the absence of Akt

signaling IRX-2 was unable to restore Bcl-2, FLIP, Bax and Bim expression (Fig. 2c). Thus, an intact PI3K/Akt signaling pathway is required for IRX-2-mediated protection from apoptosis, via the up-regulation of the survival protein expression.

IRX-2-mediated protection from apoptosis requires de novo protein synthesis

To further examine the mechanisms responsible for IRX-2-mediated protection of T cells from TMV-induced apoptosis, we co-incubated T cells with IRX-2 alone or in combination with cycloheximide (CHX) and monitored expression of pro- and anti-apoptotic proteins. CHX alone did not significantly increase the percentage of annexin V^+ Jurkat cells alone or in combination with TMV (Fig. 3a, b). However, IRX-2-mediated protection of Jurkat cells was completely abrogated in the presence of CHX (Fig. 3a, b). CHX alone did not significantly influence the expression levels of anti- and pro-apoptotic proteins in T cells. However, CHX significantly blocked the FLIP and Mcl-1 up-regulation induced by IRX-2 (Supplementary Table 2). Additionally, IRX-2 blocked the TMV-induced down-regulation of both these proteins, but in the presence of CHX, this effect was drastically reduced. A similar although weaker effect of CHX was observed for Bcl-2 (Fig. 3c; Supplementary Table 2). Although IRX-2 alone did not influence the expression of the pro-apoptotic proteins Bax and Bim, it counteracted the TMV-induced up-regulation of these proteins (Supplementary Table 2). In the presence of CHX, however, IRX-2 lost the ability to prevent the up-regulation of Bax by TMV (Fig. 3c; Supplementary Table 2).

IRX-2 prevents the TMV-induced overexpression of Fas

In view of evidence that TMV express FasL [13, 18] and that activated T cells are Fas^+ [7], we hypothesized that IRX-2 could mediate T-cell protection by modulating Fas expression on their surface. The Fas-blocking antibody, ZB-4, inhibited TMV-induced cell death of Jurkat cells in a dose-dependent manner, confirming the involvement of the Fas pathway in T-cell apoptosis (Fig. 4a). The protection mediated by IRX-2 was comparable to that mediated by 10 μg ZB-4 (Fig. 4a). TMV alone increased the percentage of Fas^+ cells and the expression of Fas (MFI) on Jurkat cells or activated primary T cells (Fig. 4b, c). IRX-2 treatment alone did not alter Fas expression on Jurkat cells, but it significantly decreased it on activated primary $CD8^+$ and $CD4^+$ cells. More important, IRX-2 pre-treatment prior to TMV addition significantly blocked Fas up-regulation in all T cells. TMV and IRX-2 had little or no effect on the expression of pro-apoptotic FasL (data not shown). Thus, one of the mechanisms of protection mediated by IRX-2 could be Fas down-regulation on the T-cell surface, making these cells more resistant to killing mediated by Fas-bearing TMV.

Overexpression of FLIP enhances the protective effect of IRX-2

cFLIP, a dominant-negative inhibitor of caspase 8, is known to be a central modulator of Fas-induced apoptosis [19, 20]. Previously, we showed that IRX-2 counteracted the TMV-induced down-regulation of FLIP expression [15]. It now appears that IRX-2 alone increases FLIP expression, an effect which can be blocked by co-incubation with CHX (Supplementary Table 2). Thus, in addition to Akt/PI3K, FLIP may be an important mediator of IRX-2 anti-apoptotic activity [19]. To test the possibility that FLIP overexpression enhances IRX-mediated protection from TMV-induced apoptosis, control (mock-vector transfected) and cFLIP-transfected Jurkat cells (Jurkat-FLIP) were analyzed for caspase activation, annexin V binding and cytochrome c release after TMV and IRX-2 treatment. FLIP-transfected Jurkat cells were found to be significantly less sensitive to TMV-induced apoptosis (Fig. 5b–d). Fewer cFLIP-transfectants than mock transfectants were annexin V^+ or Casp-$zVAD^+$ (Fig. 5b) after TMV-incubation. IRX-2 protection was significantly stronger in the cFLIP-transfected Jurkat cells, particularly after 24 h co-incubation with TMV (Fig. 3c), reducing cell death by 50% (Anx V binding) to 70% (Casp-

zVAD) in cFLIP transfectants versus around 40% of mock transfected T cells (Anx V^+ or Casp-zVAD$^+$).

An even more dramatic difference between control and cFLIP transfectants was evident upon measuring caspase activation and cytochrome c release by Western blots. As expected, TMV induced activation of caspases 8 and 9 in control cells, which coincided with the release of cytochrome c from the mitochondria into the cytosol (Fig. 5e). In contrast, Jurkat cells overexpressing cFLIP were nearly completely resistant to TMV-induced cytochrome c release and caspase activation. Interestingly, the difference in sensitivity was not only limited to TMV-induced apoptosis but was also evident upon co-incubation with the CH-11 Ab. Taken together, these findings indicate that FLIP overexpression in Jurkat cells increases their resistance to Fas-mediated apoptosis induced by TMV. Therefore, by its potential to directly boost cFLIP expression in T cells, IRX-2 protects these cells against tumor-induced death.

IRX-2 induces NF-κB translocation in Jurkat cells

Activated NF-κB proteins provide important signals for cell survival and proliferation of T cells [21, 22]. Our in vitro experiments showed that IRX-2 as well as TMV induced NF-κB activation which was comparable to that mediated by TNF-α in Jurkat cells. In the presence of both IRX-2 and TMV, p65 translocation to Jurkat cell nuclei was equally significantly up-regulated relative to control cells (Fig. 6), suggesting that TMV-mediated apoptosis as well as IRX-2-mediated protection from TMV-induced apoptosis are dependent on the NF-κB pathway activation and that additional signals might be necessary to shift the balance toward protection.

Discussion

One of the mechanisms responsible for the dysfunction of immune cells in cancer patients is the targeted apoptosis of $CD8^+$ effector T cells mediated by TMV [13, 18]. IRX-2, a primary lymphoid cell-derived biologic agent containing physiological quantities of IL-1, IL-2, IL-6, IL-8, IL-10, G-CSF, IFN-γ and TNF-α and produced under cGMP standards from stimulated human PBMC, has been shown to effectively counteract this TMV-induced T cell apoptosis [15]. We reported earlier that TMV induce apoptosis of activated T cells through induction of the receptor-mediated and mitochondrial death pathways causing activation of caspase 9, loss of mitochondrial membrane potential, release of cytochrome c and changes in the expression of mitochondria-associated proteins [15]. The pre-treatment of T cells with IRX-2 blocked all these events, indicating that IRX-2 was able to mediate protection from extrinsic and intrinsic apoptosis pathways.

While TMV-induced apoptosis of T cells is mostly a caspase-dependent phenomenon, it is accompanied by a reduction of the anti-apoptotic and a concomitant up-regulation of the pro-apoptotic Bcl-2 family members. The balance between these proteins is crucial for the fate of T cells responding to various death stimuli [23, 24]. IRX-2 restores the TMV-induced imbalance of pro- and anti-apoptotic Bcl-2 proteins (Fig. 1b, c). On the one hand, IRX-2 prevents the proteosomal cleavage of the Bcl-xL and Mcl-1 induced by TMV-driven activation of caspase 8. On the other hand, it also independently increases the basal expression of Bcl-2, FLIP and Mcl-1, thereby promoting the initial resistance of T cells to apoptosis. In conformity with other studies [20], the IRX-2-mediated up-regulation of anti-apoptotic Bcl-2 and FLIP and the down-regulation of pro-apoptotic Bax and Bim was Akt-dependent in our experiments. Other studies report that protective functions of survival cytokines such as IL-2, IL-7, IL-15 or IL-21, are largely dependent on the maintenance of a favorable balance between the Bcl-2 family members [25–30]. Our data suggest that survival cytokines tend to lower the Bax/Bcl-2 ratio and diminish sensitivity to apoptosis of

freshly harvested PBMC in cancer patients. Interestingly, the IRX-mediated modulation of Bcl-2 protein and cFLIP expression was blocked upon CHX pre-treatments. Moreover, CHX abrogated the cytoprotective effects of IRX, suggesting the requirement of protein neosynthesis for its function.

In addition to promoting T-cell survival by restoring the balance among the Bcl-2 family members, IRX-2 also uses cFLIP to mediate its cytoprotective effects. cFLIP, a well-known inhibitor of the extrinsic apoptotic pathway, through its structural homology interferes with the activation of caspase 8 [31]. Retrovirally mediated overexpression of cFLIP in activated T cells blocks Fas-induced cell death [32]. In our experiments, FLIP-transfected Jurkat cells were significantly more resistant to MV-induced apoptosis and more responsive to IRX-mediated protection than mock-transfected cells. Since cFLIP not only inhibits apoptosis by blocking caspase 8 activation, but also through inducing NF-κB activation by its N terminal cleavage products p43-cFLIP and p22-cFLIP [33, 34], we measured NF-κB translocation to the nucleus in IRX-2-treated T cells. As expected, IRX-2 induced NF-κB activation, probably not only due to up-regulated cFLIP expression, but also due to IRX-2-mediated activation of the PI3K/Akt pathway [17, 20]. However, TMV also activated NF-κB, and the pre-treatment of T cells with IRX followed by TMV induced no additional changes in the p65 translocation.

In summary, the pre-treatment of T cells with IRX-2 provides a potent protection from tumor-induced cell death. Through the release of FasL-bearing TMV, tumor cells can induce the extrinsic apoptotic pathway, and also—through cleavage of Bid—the intrinsic mitochondrial pathway. IRX-2 blocks this apoptotic signaling cascade at various levels. First, it interferes with the triggering of the receptor-mediated pathway through down-regulation of Fas expression on the T-cell surface. Second, it interrupts the transmission of the apoptotic signal from the CD95 DISC by increasing cFLIP expression, which enhances cFLIP-mediated inhibition of caspase 8 and prevents not only further activation of the extrinsic apoptotic pathway, but also cleavage of Bid, thereby blocking the initiation of the mitochondrial pathway. Finally, through the up-regulation of anti-apoptotic and down-regulation of pro-apoptotic Bcl-2 family members, IRX-2 provides additional protection from the intrinsic mitochondrial pathway. IRX-2 mediated regulation of cFLIP and Bcl-2 proteins is under the control of the Akt signaling pathway, but may not directly involve NF-κB activation and requires the neosynthesis of one or more unknown survival proteins. Moreover, induction by IRX-2 of the PI3K/Akt and NF-κB pathway may also activate additional survival-promoting proteins, rendering T cells more resistant to TMV-induced cell death. Thus, IRX-2-mediated protection appears to be a generalized phenomenon, allowing effector T cells to overcome the immunosuppressive mechanisms of the tumor microenvironment. The incorporation of IRX-2 into future cancer immunotherapies could improve their effectiveness by promoting survival of effector T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TMV Tumor-derived microvesicles

ANXV	Annexin V
PBMC	Peripheral blood mononuclear cells
CHX	Cycloheximide
BSA	Bovine serum albumin
FBS	Fetal bovine serum
MMP	Mitochondrial membrane potential
AICD	Activation-induced cell death
CTL	$CD8^+$ cytotoxic T lymphocytes
TCR	T cell receptor
FLIP	Cellular caspase 8 (FLICE)-like inhibitory protein

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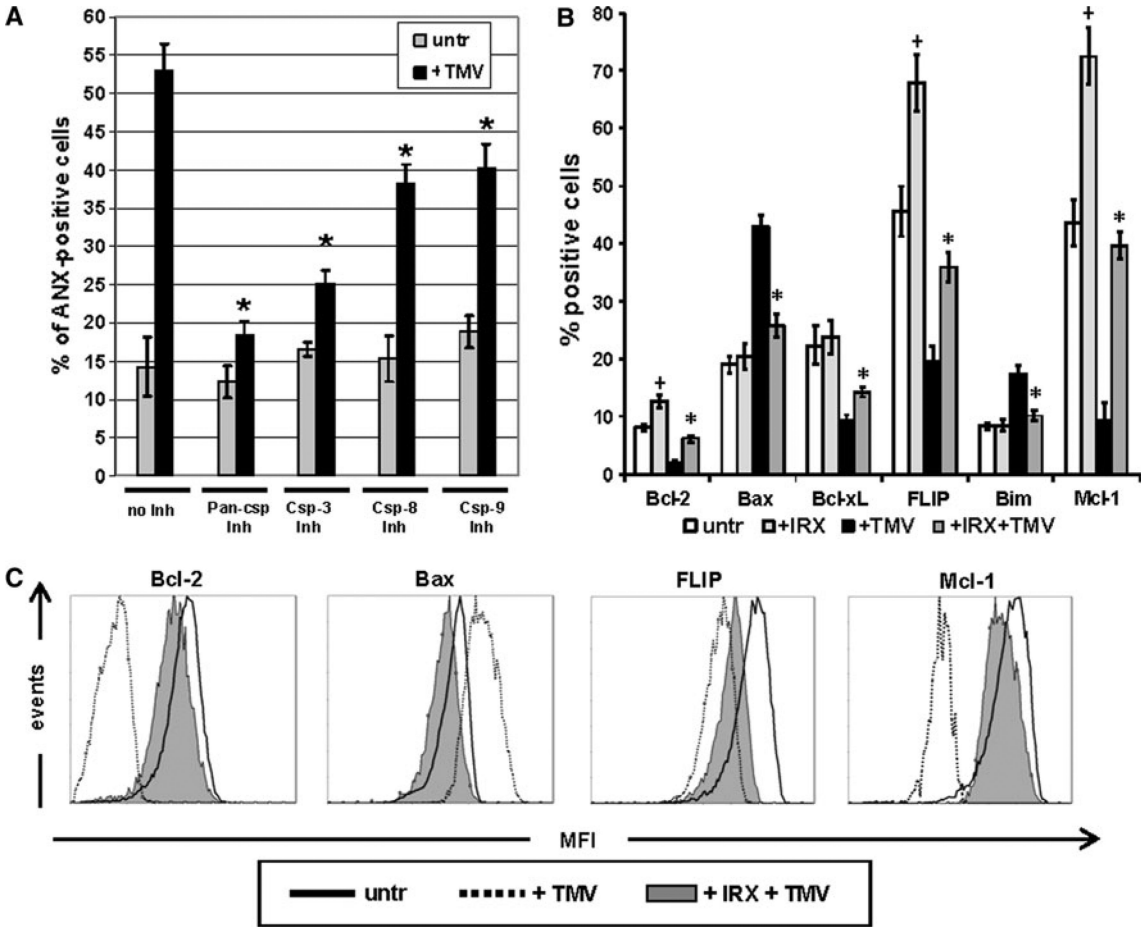

Fig. 1.

TMV-induced apoptosis of $CD8^+$ Jurkat-cells is caspase-dependent and modulates the pro- and anti-apoptotic protein expression in T cells. **a** $CD8^+$ Jurkat cells were pre-incubated with different caspase inhibitors (10 μM) for 1 h and were left untreated or were treated with TMV (10 μg) for 4 h. Cells were analyzed for annexin V binding by flow cytometry. Dead cells ($7\text{-}AAD^+$) were excluded, and the gate was set on $7\text{-}AAD^{neg}$ $CD8^+$ Jurkat cells. Results are mean percentages ± SD of annexin $V^+/7\text{-}AAD^{neg}$ cells from three independent experiments. **b** Activated peripheral blood (PB) $CD8^+$ cells were pre-incubated with IRX-2 (at 1:3 dilution) for 24 h and then treated with 10 μg TMV for additional 24 h. Expression levels of different pro- and anti-apoptotic protein were measured by quantitative flow cytometry. The data are the mean fluorescence intensity (MFI) ± SD of pro- and anti-apoptotic proteins in TMV- and IRX-2-treated activated primary $CD8^+$ cells and were obtained in 3 independent experiments (*$p < 0.005$ compared to MV-treated samples and +, $p < 0.001$ compared to untreated samples). **c** Representative histograms showing the expression (MFI) of pro- and anti-apoptotic proteins in MV- and IRX-2-treated activated primary $CD8^+$ cells

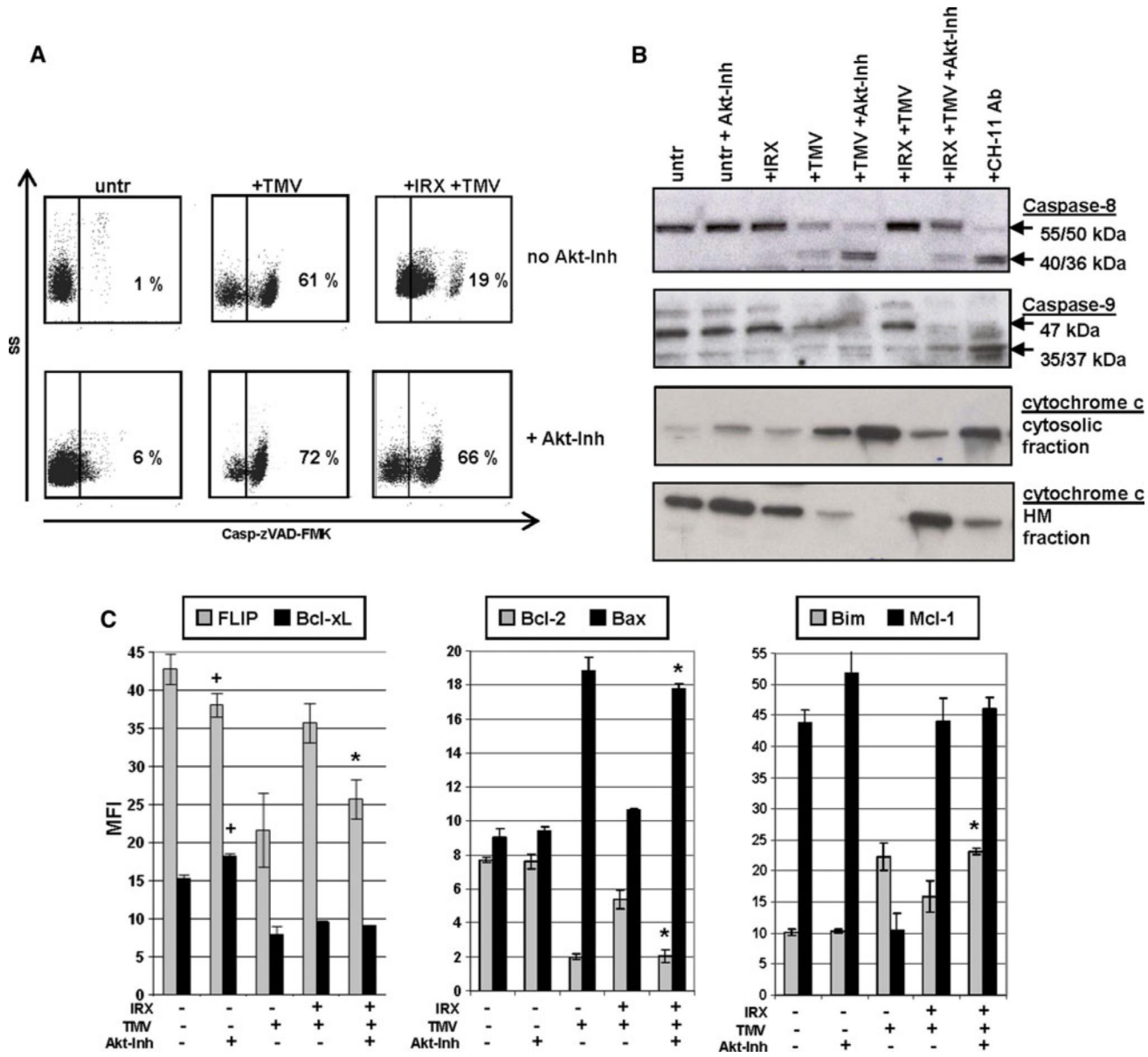

Fig. 2.

Akt involvement in IRX-2 protective effects. $CD8^+$ Jurkat cells were pre-incubated with IRX or left untreated. Then cells were treated with the Akt inhibitor Akti-1/2 (5 μM) for 1 h prior to the addition of TMV for additional 3 h. Activation of caspases in these cells was measured by flow cytometry (**a**) and Western blots (**b**). Results shown in **a** and **b** are representative of 3 independent experiments. Influence of the Akt inhibitor on the expression of pro- and anti-apoptotic proteins was measured by flow cytometry (**c**). The data are mean fluorescence intensities (MFI) ± SD of 3 independent experiments (+$p < 0.05$ in comparison to untreated samples without Akt inhibitor; *$p < 0.02$ in comparison to samples +IRX-2 and TMV without the Akt inhibitor)

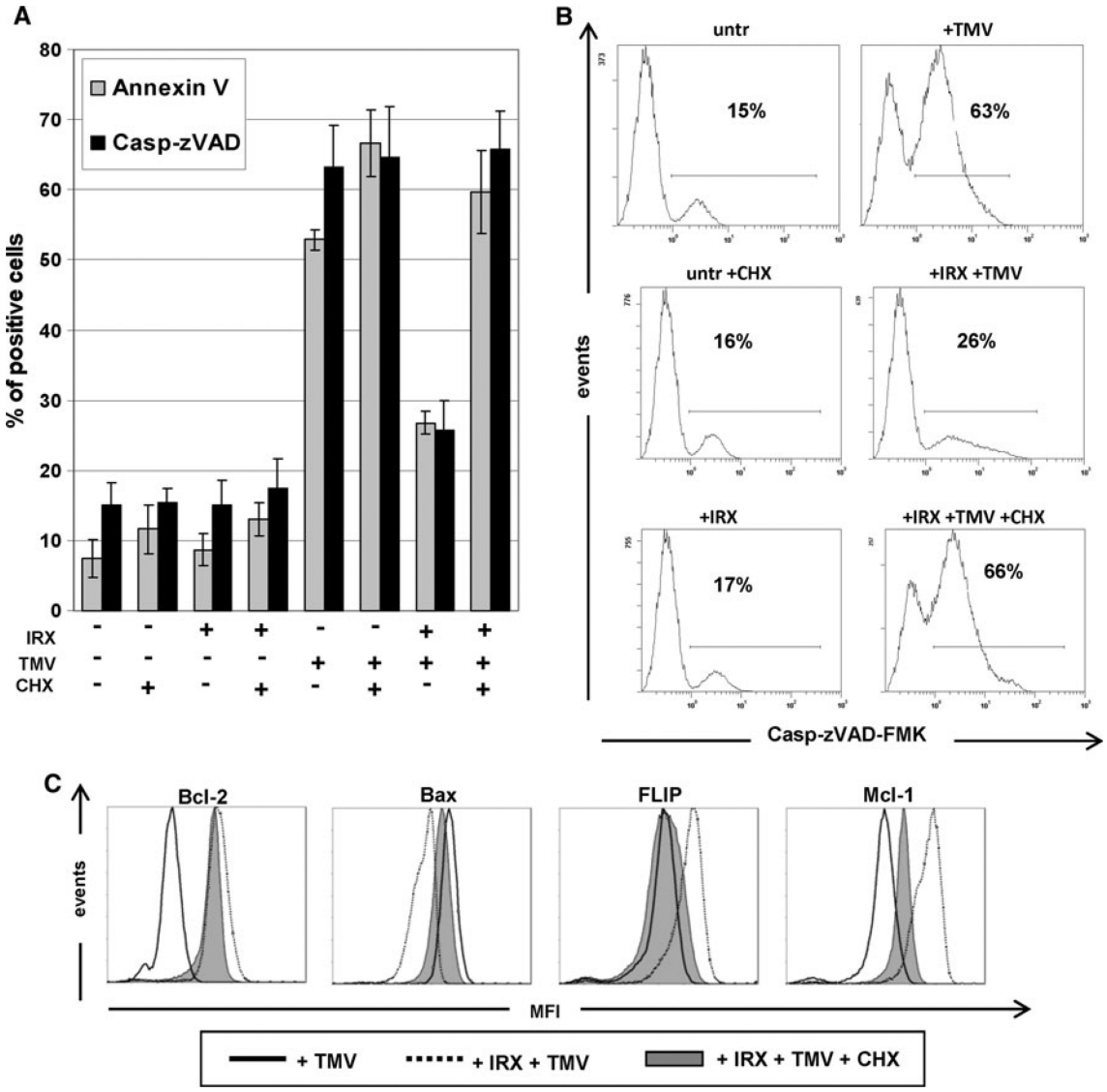

Fig. 3.

Cycloheximide abrogates IRX-2-induced protection from apoptosis. $CD8^+$ Jurkat cells were pre-incubated with IRX-2, with CHX alone or with IRX-2 + CHX (0.1 μg/mL) for 24 h and then incubated with TV for 4 h and analyzed by quantitative flow cytometry. **a** Mean percentages ± SD of annexin V positive/7AAD-negative (*gray bars*) or Casp-zVAD-FMK positive (*black bars*) $CD8^+$ Jurkat cells of 3 independent experiments. **b** Caspase-activation in MV/IRX-treated $CD8^+$ Jurkat cells after CHX pre-incubation. Representative histograms of 3 independent experiments are shown. **c** Mean fluorescence intensities (MFI) ± SD of pro-and anti-apoptotic proteins in $CD8^+$ Jurkat cells treated with MV and pre-incubated with IRX-2 ± CHX. Representative histograms out of 3 independent experiments are shown

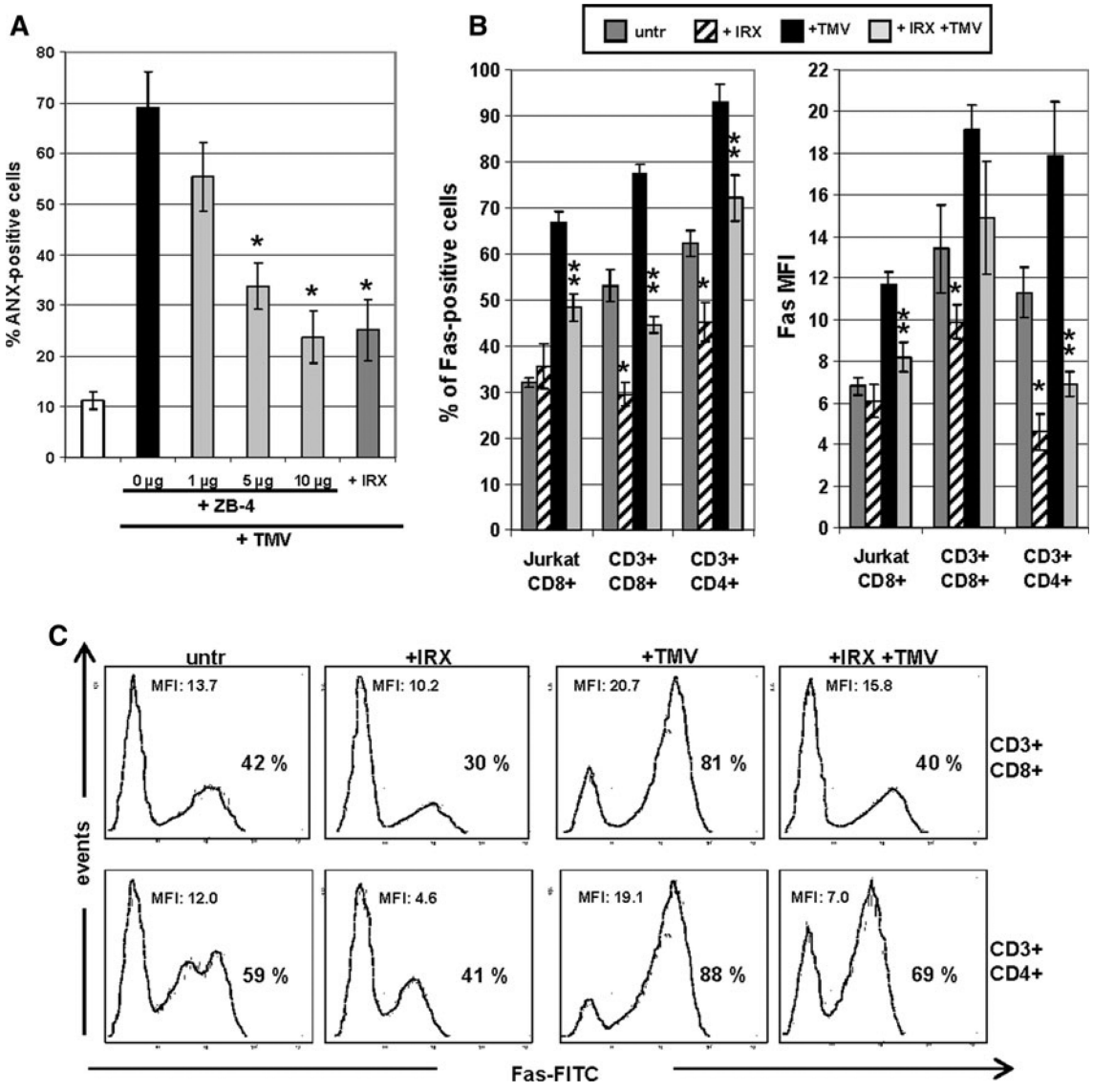

Fig. 4.

Expression of Fas in IRX-2 and/or TMV-treated T cells. **a** $CD8^+$ Jurkat cells were left untreated or were incubated with different concentrations of ZB-4 prior to addition of TV for 3 h. Cells were analyzed for annexin V binding by flow cytometry. Results are mean percentages ± SD of annexin $V^+/7\text{-}AAD^{neg}$ cells from 3 independent experiments (*$p <$ 0.001 in comparison with the TMV-treated samples). **b** $CD8^+$ Jurkat cells or activated primary $CD8^+$ or $CD4^+$ T cells were pre-incubated with IRX-2 for 24 h and then treated with 10 µg TMV for additional 4 h or 24 h. Percentages of Fas^+ cells (*left panel*) and MFI for Fas (*right panel*) were measured by flow cytometry. The data are means ± SD of 3 independent experiments (*$p < 0.02$ in comparison to untreated samples: **$p < 0.02$ in comparison to TMV-treated samples). **c** Representative histograms showing Fas-expression in activated $CD8^+$ and $CD4^+$ T cells after a TMV ± IRX-2 treatment

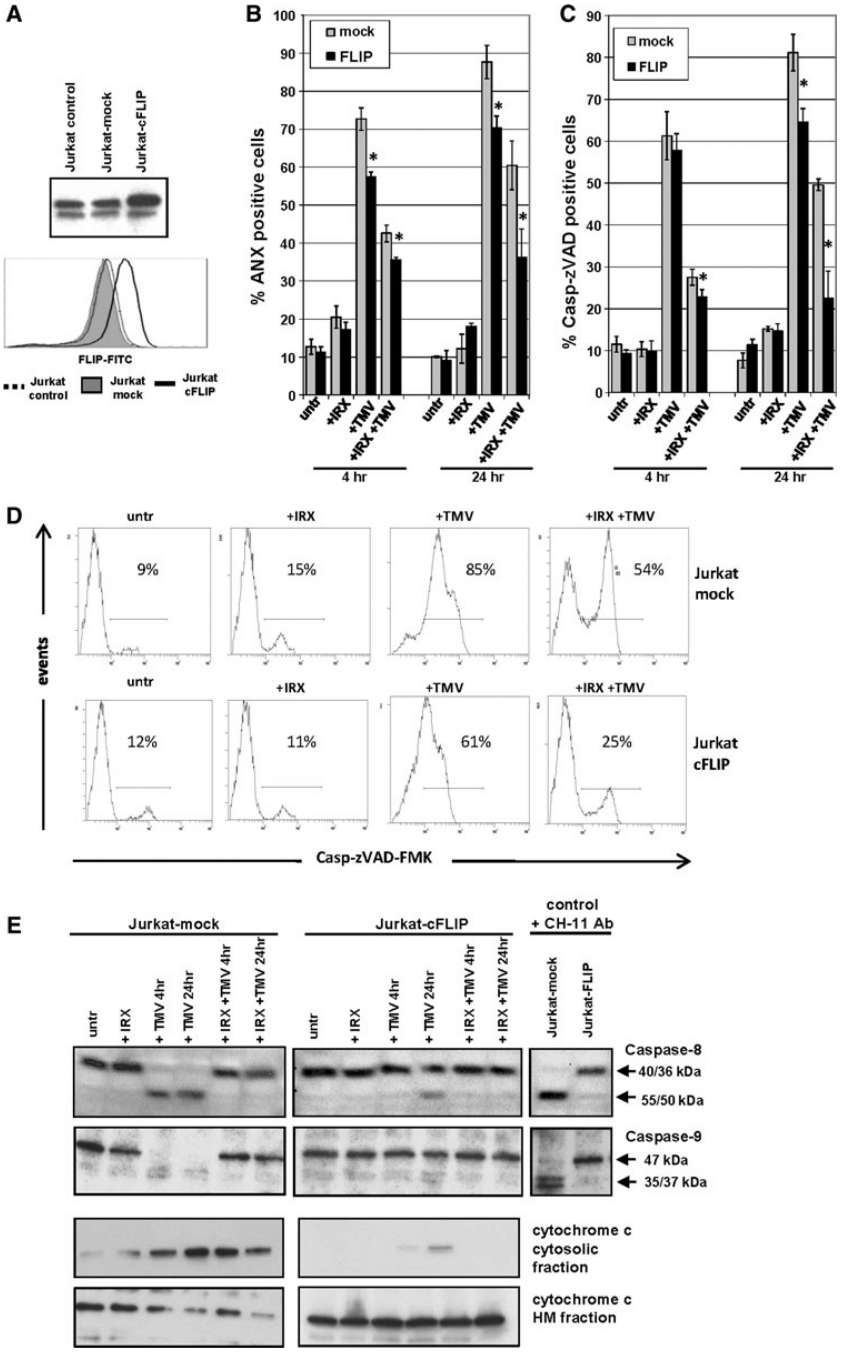

Fig. 5.

FLIP overexpression in T cells enhances IRX-2 protective effects Jurkat cells stably transfected with human FLIP (*FLIP*) or control vector (*mock*) were pre-incubated with IRX-2 or left untreated and then incubated with MV for 4 h or 24 h. **a** Overexpression of c-FLIP in Jurkat-FLIP transfectants is shown by Western blot and flow cytometry. Percentages of annexin $V^+/7AAD^{neg}$ (**b**) and of Casp-zVAD-FMK^+ (**c**) in Jurkat-FLIP transfectants and control cells after incubation with TMV and IRX-2. Results shown in **a** and **b** are means ± SD of 3 independent experiments (*$p < 0.02$ compared to mock-transfected Jurkat cells). **d** Representative histograms showing caspase-activation in TMV-

and IRX-2-treated Jurkat cells transfected with FLIP or the control vector. **e** Caspase 8 and 9 activation and cytochrome c release as measured by Western blots in TMV/IRX-2 treated Jurkat-mock or Jurkat-FLIP transfectants. Cells treated with CH-11 antibody (400 ng/mL) were used as positive controls. Note the absence of caspase-cleavage in the Jurkat-FLIP transfectants in comparison to control cells after TMV or CH-11 Ab treatment

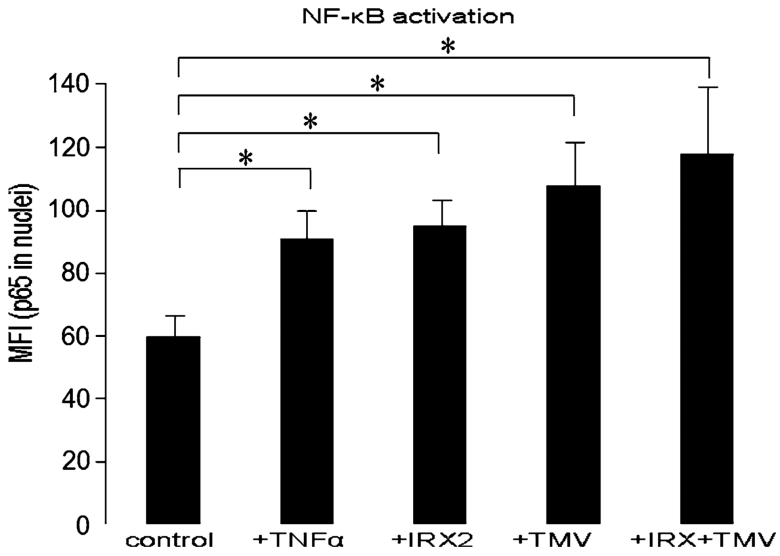

Fig. 6.

Protective effects of IRX-2 and apoptosis-inducing effects of TMV involve NF-κB activation in Jurkat cells. Translocation of p65 subunit of NF-κB from cytoplasm into nuclei was measured using an ArrayScan microscope and quantitated as MFI of p65 subunit translocated to cell nuclei. Cells were treated with medium (control), cytokines or TMV as described in "Materials and methods". The data are means ± SD from 3 independent experiments. *Significant differences from untreated controls at $p < 0.05$