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Regulation of apoptosis by type III interferons

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Abstract. *Objective*: Two types of interferons (IFNs), type I (IFN-α/β) and type III (IFN-λs), utilize distinct receptor complexes to induce similar signalling and biological activities, including recently demonstrated for IFN-λs antitumour activity. However, ability of type III IFNs to regulate cell population growth remains largely uncharacterized. *Materials and methods*: Intact and modified human colorectal adenocarcinoma HT29 cells were used to study regulation of apoptosis by IFN-λs. *Results and Conclusions*: We report that the IFN-λR1 chain of the type III IFN receptor complex possesses an intrinsic ability to trigger apoptosis in cells. Signalling induced through the intracellular domain of IFN-λR1 resulted in G_1/G_0 phase cell cycle arrest, phosphatidylserine surfacing and chromosomal DNA fragmentation. Caspase-3, caspase-8 and caspase-9 were activated; however, pancaspase inhibitor Z-VAD-FMK did not prevent apoptosis. In addition, the extent of apoptosis correlated with the level of receptor expression and was associated with prolonged IFN-λ signalling. We also demonstrated that the ability to trigger apoptosis is a unique intrinsic function of all IFN receptors. However, more robust apoptosis was induced by signalling through type III IFN receptor than through type I or type II (IFN-γ) receptors, suggesting higher cytotoxic potential of type III IFNs. In addition, we observed that IFN-γ treatment sensitized HT29 cells to IFN-λ-mediated apoptosis. These results provide evidence that type III IFNs, alone or in combination with other stimuli, have the potential to induce apoptosis.

INTRODUCTION

Three types of interferons (IFNs), type I, type II and type III, are important mediators of an antiviral response (Meager 2006). IFNs induce an antiviral state in cells, up-regulate major histocompatibility complex (MHC) antigen expression, modulate proliferation and apoptosis, and stimulate host immune responses. Each type of IFN signals through a unique receptor complex. All type I IFNs (13 IFN-α proteins and one of each IFN-β, IFN-ω, IFN-κ and IFN-ε in humans) signal through a common receptor complex composed of IFN-αR1 and IFN-αR2 subunits (Kotenko & Langer 2004; Pestka *et al*. 2004; Huang *et al*. 2007). Type II IFN, IFN-γ, exerts its biological activities through an IFN-γ receptor complex consisting of IFN-γR1 and

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IFN-γR2 chains. IFN-λR1 and interleukin (IL)-10R2 receptors pair to form a functional receptor complex for recently identified type III IFNs (Kotenko *et al.* 2003) (IFN-λ1, IFN-λ2 and IFN-λ3, also designated as IL-29, IL-28A and IL-28B, respectively; Sheppard *et al.* 2003). IL-10R2 can also combine with either IL-10R1, IL-20R1 or IL-22R1 to yield receptor complexes for cytokines of the IL-10 family, IL-10 itself, IL-26 and IL-22, respectively (Kotenko *et al.* 1997, 2001; Xie *et al.* 2000; Hor *et al.* 2004; Sheikh *et al.* 2004). Receptors for IFNs and IL-10-related cytokines demonstrate low sequence similarity in their extracellular domains and comprise the class II cytokine receptor family (CRF2) (Kotenko 2002; Kotenko & Langer 2004; Pestka *et al.* 2004). IFNs and IL-10-related cytokines also share limited amino acid similarity (Renauld 2003; Kotenko & Langer 2004).

The Janus kinase (JAK)–signal transducers and activators of transcription (STAT) pathway is the major signal transduction pathway activated by IFNs and IL-10-related cytokines (Renauld 2003; Kotenko & Langer 2004; Pestka *et al.* 2004). Activation of type I and type III IFN receptors, despite their differences, results in similar signalling cascades that include phosphorylation of JAK kinases JAK1 and Tyk2 followed by phosphorylation of STAT1 and STAT2, as well as STAT3, STAT4 and STAT5 to a lesser extent (Kotenko *et al.* 2003; Dumoutier *et al.* 2004; Kotenko & Langer 2004; Pestka *et al.* 2004). STATs homo- or heterodimerize, translocate to nuclei, and bind to the promoter region of IFN-stimulated genes leading to downstream gene transcription. STAT homo- and heterodimers bind to specific DNA elements. For instance, STAT1–STAT2 heterodimers associate with a DNA-binding protein IRF9 (p48) forming a transcriptional complex designated IFN-stimulated gene factor 3, which binds to the IFN-stimulated response element. Type II IFN predominately activates STAT1 and transcription of genes preceded by an IFN-γ-activated sequence element. STAT3 is a major STAT activated in response to IL-10-related cytokines. Cross-talk between the JAK-STAT pathway and other signalling pathways, such as phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathways, modulates the array of cellular responses to IFNs and IL-10-related cytokines (Renauld 2003; Platanias 2005).

Type I IFNs are used therapeutically for treatment of chronic viral infections and various cancers. Recently, antitumour potential of IFN-λs was demonstrated in the mouse B16 melanoma and fibrosarcoma models (Lasfar *et al.* 2006; Sato *et al.* 2006; Numasaki *et al.* 2007). As anticancer agents, IFNs activate both immune and non-immune mechanisms towards tumour destruction (Borden 2005). IFNs target immune cells to enhance an antitumour responses (Dunn *et al.* 2004, 2005). IFNs also affect tumour stromal cells and suppress angiogenesis, events that are required to maintain tumour survival and proliferation *in vivo* (Lindner 2002; Lasfar *et al.* 2006; Meager 2006). IFNs can also act directly on tumour cells by inhibiting their proliferation and inducing apoptosis alone or in combination with other stimuli (Borden 2005; Pokrovskaja *et al.* 2005).

Apoptosis is primarily executed by caspases, the cysteine aspartate-specific proteases (Thornberry & Lazebnik 1998; Stennicke & Salvesen 2000). Two major pathways, one mediated by mitochondria (intrinsic pathway) and another mediated by death receptors (extrinsic pathway), lead to activation of caspases (Hengartner 2000; Clemens 2003; Kalvakolanu 2004). Members of the TNF family of ligands bind to death domain containing receptors to initiate a signalling cascade leading to caspase cleavage. In addition, DNA damage, cytotoxic drugs, or the absence of cytokines which function as survival factors, induce functional changes in mitochondria, resulting in caspase activation. Activated caspases cleave various target proteins causing genomic DNA fragmentation, changes in cell morphology and, eventually, cell death.

Although type I IFNs are capable of inducing apoptosis in tumour cells, molecular mechanisms mediating a cell death in response to IFNs remain to be further characterized. By binding to their corresponding cell receptor complexes, IFNs induce quick and potent signalling which leads to

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expression of more than 300 IFN-stimulated genes (ISGs; Der *et al*. 1998; Doyle *et al*. 2006; Marcello *et al*. 2006). Many ISGs encode proteins that have been implicated in apoptosis (Clemens 2003; Kalvakolanu 2004; Borden 2005). Apoptotic stimulatory abilities of type III IFNs have not been previously described. Although downstream signalling events and several biological activities induced by IFN-λs are nearly the same as that of type I IFNs (Kotenko *et al*. 2003; Dumoutier *et al*. 2004; Kotenko & Langer 2004), cellular targets of type I and type III IFNs differ. Whereas type I IFN receptors are expressed in most cell types, IFN-λR1 demonstrates a more restricted pattern of expression, limiting the IFN-λ response primarily to epithelium-like tissues (Doyle *et al*. 2006; Lasfar *et al*. 2006; Zhou *et al*. 2007). Because most solid cancers have epithelial origin, it is of interest to investigate whether type III IFNs can induce apoptosis in cancer cells, and, therefore, may have a potential for cancer therapy. Here, we demonstrate that induction of IFN-λ signalling in colorectal adenocarcinoma HT29 cells leads to caspase activation, externalization of phosphatidylserine (PS), and DNA fragmentation, eventually resulting in cell death.

MATERIALS AND METHODS

Plasmid construction

Plasmids, pEF-FL-10R1/λR1, pEF-FL-IL-10R1/αR2c and pEF-FL-10R1/20R1, have been described previously (Kotenko *et al*. 2003; Sheikh *et al*. 2004). Fragments containing the transmembrane and intracellular domains of human IFN-γR1 and IL-22R1 were excised from plasmids pEF3-γR1/γR1 and pEF-22R1 (CRF2-9), respectively (Kotenko *et al*. 1999, 2001), with the use of *Nhe*I and *Bss*HII restriction endonucleases, and were cloned into corresponding sites of plasmid pEF-FL-10R1/λR1, resulting in pEF-FL-10R1/γR1 and pEF-FL-10R1/22R1, respectively. A DNA fragment encoding the extracellular domain of IFN-λR1 was amplified by PCR with primers 5′-GCCGGATCCCCGTCTGGCCCCTCCCCAGAA-3′ and 5′-AGGGCTAG CCAGTTGGCTTCTGGGACCTCC-3′ and plasmid pEF-CRF2-12/γR1 (λR1/γR1) (Kotenko *et al*. 2003) as template. The resulting PCR product was cloned into plasmid pEF-FL-10R1/λR1 with the use of *Bam*HI and *Nhe*I restriction endonucleases, resulting in plasmid pEF-FL-IFN-λR1. Nucleotide sequences of the modified regions of all constructs were verified in their entirety by DNA sequencing.

Cells, transfection and flow cytometry

Human colorectal adenocarcinoma HT29 cells and human myeloma U266 cells were maintained in RPMI 1640 medium (Sigma-Aldrich, Santa Louis, MO, USA) with 10% heat-inactivated foetal bovine serum (FBS; Sigma-Aldrich). HT29 cells were transfected with expression plasmids with the use of TransIT transfection kit (Mirus, Madison, WI, USA), and G418-resistant transfectants were selected in media containing antibiotic G418 (400 μg/mL).

Flow cytometry with FLAG, or W6/32 antibody (Ab) was performed to evaluate expression of FLAG-tagged receptors, or MHC class I antigen molecules (HLA-B7), respectively, on the cell surface as described previously (Kotenko *et al*. 2003). Flow cytometry was also used to determine activation of caspase-3, caspase-8 and caspase-9 with antibodies specific for active forms of caspases (caspase-3 Ab, BD Biosciences, San Jose, CA, USA; caspase-8 Ab and caspase-9 Ab, Cell Signalling Technology, Danvers, MA, USA), and PS surfacing with FITC-conjugated annexin V (BD Biosciences), following manufacturers' suggested protocols. To determine integrity of the cell membrane, annexin V-FITC-stained cells were also treated with propidium iodide (PI)

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(8 mg/L). Flow cytometry was also used to detect changes of CD95/Fas expression levels on the cell surface with CD95/Fas antibodies (Clones M3 and M38; ATCC numbers HB11465 and HB11726, respectively).

Cell cycle analysis and TUNEL assay

To determine distribution of cells through the cell cycle, 10^5–10^6 cells (attached and floating) were collected, rinsed with phosphate-buffered saline (PBS) with 5% FBS, permeabilized by incubation with 0.1% Triton X-100 at 22 °C for 5 min, washed with PBS with 5% FBS, precipitated by centrifugation at 2000 **g**, 4 °C, and re-suspended in PBS with 5% FBS containing 8 mg/L PI and 40 mg/L RNase A at 22 °C for 10 min in the dark. Cell distribution through the cell cycle was analysed by flow cytometry.

TUNEL assay to determine DNA fragmentation in cells undergoing apoptosis was performed according to the manufacturer's suggested protocols (Promega, Madison, WI, USA). Briefly, $3–5 \times 10^6$ cells were collected by trypinization, washed twice with cold PBS, fixed in 4% paraformaldehyde at 4 °C for 20 min, washed again with PBS, and permeabilized with 0.5 mL 0.5% saponin at 22 °C for 5 min. After another PBS wash, cells were incubated with 80 μL equilibration buffer at 22 °C for 5 min, and washed with PBS, and 50 μL Nucleotide Mix was added and incubated with each cell sample in the dark at 37 °C for 1 h. Cells were washed again with PBS and were analysed using fluorescence microscopy. DAPI (4′,6-diamino-2-phenylindole dihydrochloride) staining (Calbiochem, San Diego, CA, USA) was used to localize cell nuclei.

To induce apoptosis, HT29 cells were treated with the combination of IFN-γ (10 ng/mL) and TNF-α (1 ng/mL), and HT29/FL-10R1/λR1 cells were treated with IL-10 (10 ng/mL) at 37 °C for 48 h. In other apoptosis-related assays, various cytokine concentrations and time points of treatment were used as indicated in the text. In various assays, caspase inhibitor Z-VAD-FMK (Calbiochem) was used at concentration 30 μM or as described in the text.

Proliferation and cell viability assays

To determine cell proliferation, an equal number of cells (10^5/well) were plated in wells of 6-well plates and treated with various concentrations of cytokines, as indicated in the text. Floating cells were collected and combined with adherent cells released from the wells by trypsinization, and cell counts were performed.

To determine cell viability, an equal number of cells (3×10^4/well) were plated in all wells of 96-well microtitre plate and treated with various concentrations of cytokines as described in the text. Dead cells lost their attachment, and at indicated time points live adherent cells were visualized by staining with crystal violet at 22 °C for 5 min.

Electrophoresis mobility shift assays and immunoblotting

Cells were treated with cytokines for 15 min at 37 °C as indicated in the text and were used for electrophoresis mobility shift assay (EMSA) to detect activation of STAT1. EMSA was performed with a 22-bp DNA probe containing a STAT1-binding IFN-γ-activated sequence as described previously (Kotenko *et al*. 2003).

Cleavage of caspases in cells was measured by immunoblotting from whole cell lysates using anticaspase-3 antibody (BD Biosciences), anticaspase-8 antibody and anticaspase-9 antibody (Cell Signalling Technology).

Luminescence caspase activity assay

Caspase activities were determined by using Caspase-Glo-9 and Caspase-Glo-3/7 assays (Promega), following the manufacturer's suggested protocols. An equal number of cells (3×10^4 cells/

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100 μL/well) were plated in all wells of a 96-well microtitre plate and left untreated or treated with various stimuli, as described in the text. After 48 h, 100 μL of a freshly prepared mixture of Caspase-Glo reagents and buffer was added to each well. The plate was put on a shaker at 22 °C for 40 min in the dark. Luminescence was read by the Fluoroskan Ascent FL plate reader (Thermo Electron Inc., Waltham, MA, USA).

RESULTS

IFN-λ signalling induces cell death

Regulation of cell proliferation by IFNs varies and depends on cell types and specific conditions (Clemens 2003; Kalvakolanu 2004; Pokrovskaja *et al*. 2005). Type I and type II IFNs can block or promote proliferation, or induce apoptosis in cells. Recently, identified type III IFNs, IFN-λs, have also been reported to induce antiproliferative responses in selected cell types, such as human glioblastoma LN319 cells, pancreatic neuroendocrine tumour BON1 cells, and colorectal carcinoma HCT116 cells (Brand *et al*. 2005; Meager *et al*. 2005; Zitzmann *et al*. 2006).

Responsiveness of cells to IFNs is determined by the level of IFN receptor expression, and ability of IFN-λ to block cell proliferation seems to correlate with the level of IFN-λR1 expression (Meager *et al*. 2005). Overexpression of human IFN-λR1 in murine BW5147 T lymphoma cells also enables human IFN-λ1 to block proliferation of BW5147 cells (Dumoutier *et al*. 2004). We aimed to investigate the ability of type III IFNs, IFN-λs, to regulate cell proliferation and to compare it to activities of types I and II IFNs. We selected HT29 colorectal adenocarcinoma p53-deficient cells for the experiments, because these cells respond well to all three types of IFNs (Kotenko *et al*. 2003; Fig. 1). Treatment of the cells for 72 h with either type I or type III IFNs led to up-regulation of MHC class I antigen expression on the cell surface (Fig. 1a) but did not significantly affect proliferation of the cells (Fig. 1b). IFN-γ treatment, particularly at high concentration (100 ng/mL), had an inhibitory effect on cell proliferation (Fig. 1b). To investigate whether overexpression of IFN-λR1, the signal competent subunit of the IFN-λ receptor complex, will render HT29 cells more sensitive to the IFN-λ-induced antiproliferative effect, HT29 cells were transfected with FLAG epitope-tagged IFN-λR1 expression plasmid. However, ectopic expression of FL-IFN-λR1 in HT29 cells resulted in a gradual loss of the FL-IFN-λR1 expression, and clones expressing detectable levels of FL-IFN-λR1 could not be obtained (data not shown). Therefore, we used previously created chimaeric receptor FL-IL-10R1/IFN-λR1 (FL-10R1/λR1) (Kotenko *et al*. 2003) to dissect molecular mechanisms involved in the regulation of cell proliferation. Because IL-10 and IFN-λs share the second chain of their corresponding receptor complexes (Fig. 1c), treatment with IL-10 induced IFN-λ signalling in HT29 cells expressing FL-10R1/λR1 chimaeric receptors (Kotenko *et al*. 2003). To characterize biological activities and signalling mediated by FL-10R1/λR1 receptor in HT29 cells, two clonal populations expressing different levels of FL-10R1/λR1 were selected (Fig. 1d). IL-10 treatment induced a strong antiproliferative response and cell death in a dose- and time-dependent manner (Fig. 1e). The antiproliferative effect was stronger in cells that expressed FL-10R1/λR1 at a higher level. At 72 h, cells became rounded and lost their adherence in response to IL-10, suggesting induction of apoptosis (Fig. 1e and data not shown). In response to a low dose of IL-10 (0.3 ng/mL) when the antiproliferative response was minimal, cells up-regulated the level of MHC class I antigen expression (Fig. 1f), the biological activity characteristic of IFN-λ signalling (Kotenko *et al*. 2003).

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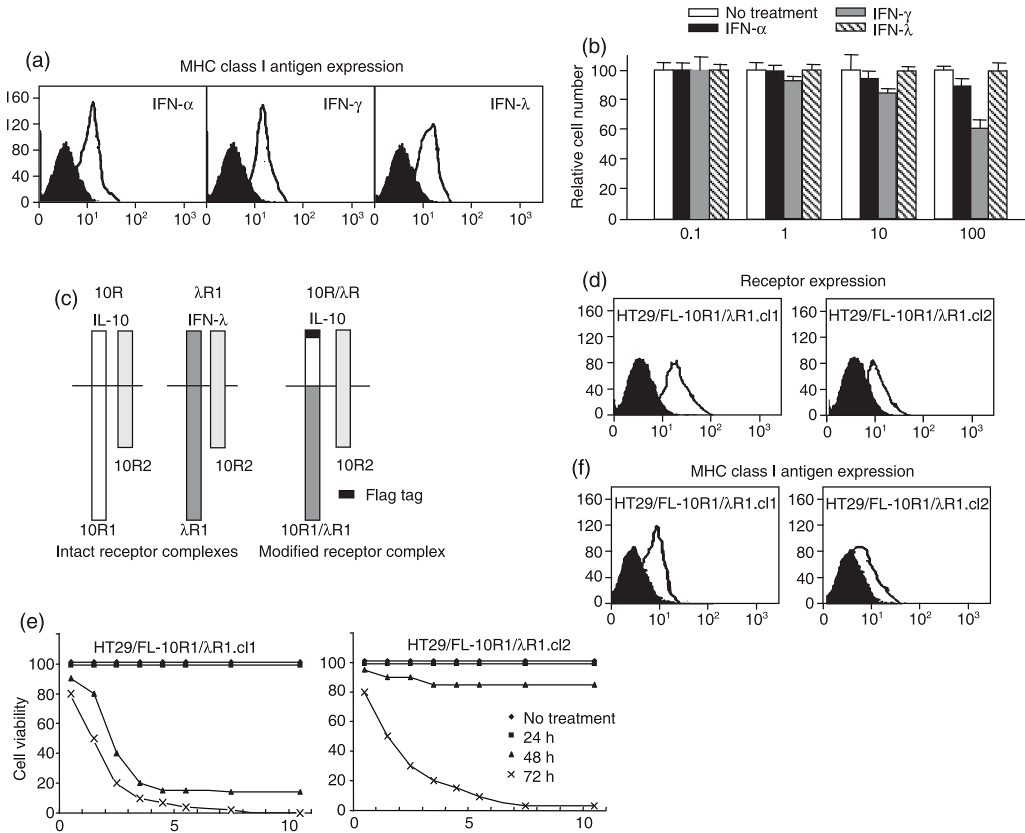

Figure 1. Response of HT29 and HT29/FL-10R1/λR1 cells to IFN signalling. (a) Major histocompatibility complex (MHC) class I antigen expression on the cell surface of HT29 cells treated for 72 h with IFNs (10 ng/mL) was determined by flow cytometry with HLA-B7 antibody [untreated cells (shaded histograms), IFN-treated cells (open histograms)]. Here and in other figures demonstrating flow cytometry results, the ordinate represents relative cell number, and the abscissa relative fluorescence in log scale. (b) HT29 cells were stimulated with various amounts of either IFN-α2 (IFN-α), IFN-γ or IFN-λ1 (IFN-λ) for 72 h and their proliferation was evaluated by cell counting shown as a percentage to control untreated cells. (c) The receptors used in this study are schematically shown. IL-10 and IFN-λ receptor complexes (10R and λR) share IL-10R2 (10R2), a receptor chain with a short intracellular domain, and also contain a unique signalling competent receptor chain with a long intracellular domain, either IL-10R1 (10R1) or IFN-λR1 (λR1) chain, respectively. Chimaeric IL-10R/IFN-λR1 (10R/λR) complex is composed of the intact IL-10R2 chain and a chimaeric 10R1/λR1 receptor subunit that has the IL-10R1 extracellular domain tagged with the FLAG epitope at the N-terminus and IFN-λR1 transmembrane and intracellular domains. (d) Expression of the FL-10R1/λR1 chain in two clonal populations of HT29/FL-10R1/λR1 cells (open histograms) in comparison with parental HT 29 cells (shaded histograms) was tested by flow cytometry with FLAG antibody. (e) Viability of the clonal cell populations was tested at different time points after the treatment with various amounts of IL-10. Here and in Fig. 3g,h, the abscissa represents concentration of IL-10 (ng/mL), and the ordinate represents percentage of live cells relative to the control untreated cells. (f) Level of MHC class I antigen expression in clonal populations of HT29/FL-10R1/λR1 cells, untreated (shaded histograms) or treated with IL-10 (0.3 ng/mL) for 72 h (open histograms), was determined by flow cytometry.

IFN-λ signalling blocks cell cycle progression

As previous research has shown that IFNs may induce growth arrest and/or apoptosis in some cell lines (Sangfelt *et al*. 1997; Thyrell *et al*. 2002), we investigated whether IFN-λ signalling would affect cell progression through the cell cycle. IL-10-stimulated HT29/FL-10R1/λR1 cells

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started to accumulate in G_0/G_1 and G_2 phases at 8 h after IL-10 treatment, as demonstrated by propidium iodide (PI) staining. A population of cells in S phase completely disappeared at 24 h, and all cells were in G_0/G_1 phase or dead at 48 h (Fig. 2a). Results demonstrate that before the loss of the adherence of HT29/FL-10R1/λR1 cells became apparent at 72 h in response to IFN-λ signalling, cell cycle progression was blocked in G_0/G_1 phase, similar to the events observed in human multiple myeloma U266 cells undergoing type I IFN-induced apoptosis (Sangfelt *et al*. 1997; Thyrell *et al*. 2002).

Redistribution of phosphatidylserine and DNA fragmentation in cells undergoing IFN-λ signalling

Numerous changes occur in cells undergoing apoptosis. These changes include the redistribution of PS to the external surface of the cell membrane, activation of caspases leading to cleavage of their target proteins, and DNA fragmentation (Hengartner 2000, 2001; Wu *et al*. 2006). Externalization of PS and DNA fragmentation assays are often used to demonstrate induction of apoptosis in cells. Appearance of PS on the cell surface of apoptotic cells can be detected by flow cytometry with FITC-labelled annexin V. To assess integrity of the cell membrane, cells were also stained with PI and percentage of annexin V-positive and PI-negative apoptotic cells was determined. The experiments demonstrated that PS externalization was induced in HT29/FL-10R1/λR1 cells in response to IL-10 treatment as early as 24 h and further increased at 48 h (Fig. 2b,c). A similar response was observed in HT29 cells treated with a combination of IFN-γ and TNF-α, that was shown to induce apoptosis in HT29 cells (Wilson & Browning 2002).

The TUNEL assay was performed to detect whether DNA fragmentation occurred in the cells. Ends of DNA fragments were fluorescently labelled by terminal deoxynucleotidyl transferase and cell fluorescence was measured. A high level of fluorescence was observed in parental HT29 cells after 48 h of IFN-γ and TNF-α treatment and in HT29/FL-10R1/λR1 cells treated with IL-10 (Fig. 2d), thus showing that IFN-λ signalling-induced cell changes characteristic of apoptosis.

Activation of caspases is induced by IFN-λ signalling

Caspases have been shown to be activated in apoptosis induced by type I and type II IFNs (Thyrell *et al*. 2002; Clemens 2003; Gamero *et al*. 2006a). Inactive caspases are present in cells as pro-enzymes that are activated by the proteolytic cleavage. To investigate whether caspases were activated in HT29/FL-10R1/IFN-λR1 cells undergoing IFN-λ signalling, cleavage of caspase-3 (the major effector caspase) was tested by flow cytometry and immunoblotting. Both assays demonstrated that the activated form of caspase-3 was detected at 24 h and the amount of activated caspase-3 was strongly increased at 36 h after stimulation, and remained at the same

Figure 2. IFN-λ signalling induces apoptosis in HT29 cells. (a) Distribution through the cell cycle was measured in HT29/FL-10R1/λR1 cells after IL-10 treatment (10 ng/mL) for 8, 24, 36 and 48 h by PI staining. (b) HT29 cells were left untreated or treated with IFN-γ (10 ng/mL) and TNF-α (1 ng/mL), and HT29/FL-10R1/λR1 cells were left untreated or treated with IL-10 (10 ng/mL) for 24 and 48 h, and binding of FITC-conjugated annexin V to phosphatidylserine was evaluated by flow cytometry. (c) Annexin V-FITC-labelled cells were also stained with PI, and percentage of apoptotic (annexin V-FITC-positive, PI-negative) cells was determined by flow cytometry. Here and in Fig. 6c, the ordinate represents percentage of apoptotic cells out of total cell number. (d) TUNEL assay was performed to detect chromosomal DNA fragmentation. Parental HT29 and HT29/FL-10R1/λR1 cells were left untreated, treated with IL-10 (10 ng/mL) for 48 h, or incubated at 60 °C for 10 min (negative control). Parental HT29 cells were also treated with IFN-γ (10 ng/mL) and TNF-α (1 ng/mL) for 48 h as a positive control. The ordinate represents fluorescence intensity shown as percentage to the control.

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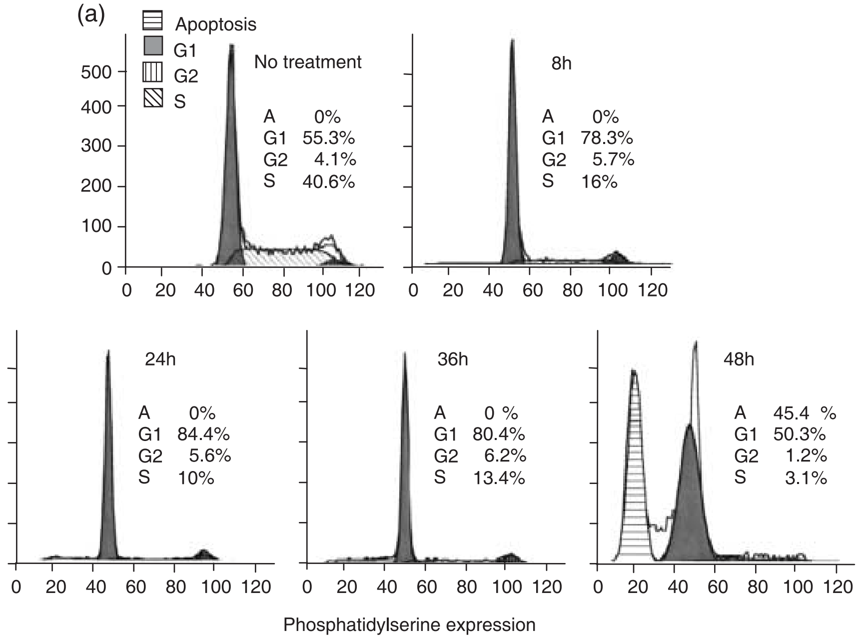

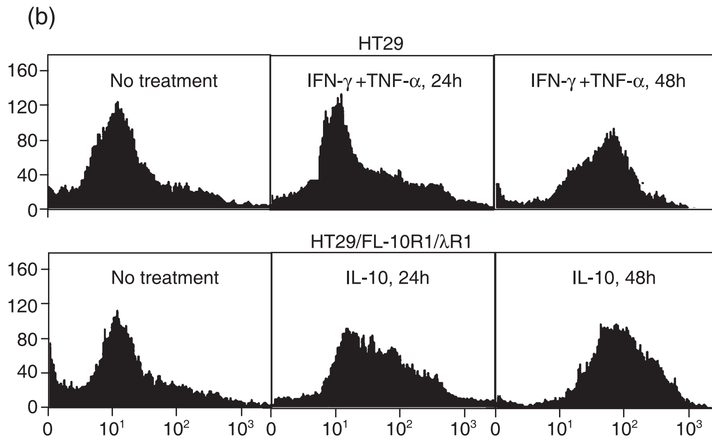

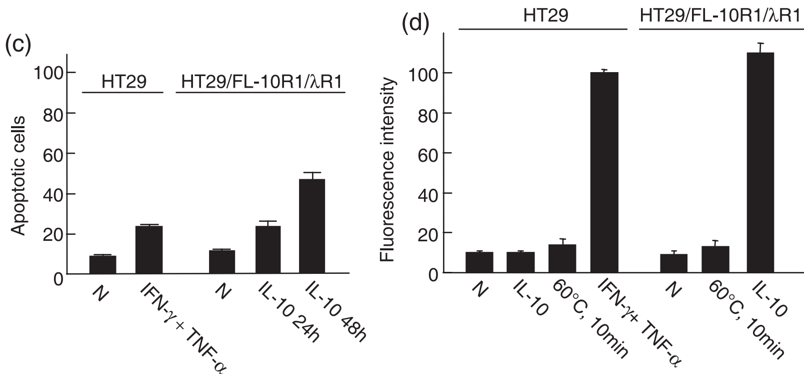

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level at 72 h (Fig. 3a and data not shown). Caspase-8, one of the major extrinsic initiator caspases, was also detected as the cleaved activated form at 24 h and its activation proceeded to further increase at 36, 48, 60 and 72 h (Fig. 3b). Caspase-9, the initiator caspase activated by stress signals in mitochondria, demonstrated an activation pattern similar to that of caspase-8 (Fig. 3c). As a control, caspase activation was also demonstrated in parental HT29 cells treated with IFN-γ and TNF-α (Fig. 3a–c).

Next, we used the pancaspase inhibitor Z-VAD-FMK to investigate whether activation of caspases was required for IFN-λ-induced apoptosis in HT29 cells. U266 cells were used to demonstrate that Z-VAD-FMK inhibited the antiproliferative response of U266 cells triggered by IFN-α treatment within 48 and 72 h as shown previously (Thyrell *et al*. 2002; Fig. 3d). Cleavage of caspase-3 in IL-10-stimulated HT29/FL-10R1/λR1 cells was blocked by Z-VAD-FMK as determined by flow cytometry (Fig. 3e); in contrast, Z-VAD-FMK was unable to block and even promoted cleavage of caspase-9 (Fig. 3e). Next, we used caspase-specific luminogenic substrates to determine whether cleaved caspases were proteolytically active (Fig. 3f). Proteolytic activity of both caspase-3 and caspase-9 was detected in HT29 cells treated with IFN-γ and TNF-α or in HT29/FL-10R1/IFN-λR1 cells undergoing IFN-λ signalling, at 48 h after stimulation. In all cases, Z-VAD-FMK treatment completely inhibited proteolytic activities of both caspase-3 and caspase-9 as measured by their inability to cleave caspase-specific luminogenic substrates (Fig. 3f). Thus, although pancaspase inhibitor Z-VAD-FMK failed to block cleavage of caspase-9, it still completely inhibited caspase-9 proteolytic activity. Nevertheless, Z-VAD-FMK treatment did not inhibit cell death induced in HT29/FL-10R1/λR1 cells by IL-10 (Fig. 3g). Therefore, it appears that inhibition of caspases did not protect the cells from IFN-λ signalling-induced cell death, and suggested that other intracellular mediators of apoptosis could contribute to IFN-λ-triggered cell death.

Continuous IFN-λ signalling is required for apoptosis of HT29 cells

Interferons can promote apoptosis by up-regulating death domain-containing receptors and their ligands, such as Apo2L/TRAIL and CD95L/FasL, in responsive cells (Clemens 2003; Kalvakolanu 2004; Pokrovskaja *et al*. 2005). However, conditioned media from IL-10-treated HT29/FL-10R1/λR1 cells undergoing apoptosis did not induce cell death in parental HT29 cells, indicating that soluble mediators are not involved (data not shown). Fas (CD95) expression was also unchanged in HT29-FL-10R1/λR1 cells treated for 48 h with IL-10 (10 ng/mL), or in parental HT29 cells treated with IFN-α or IFN-λ (100 ng/mL), whereas even a low concentration of IFN-γ (0.1 ng/mL) was sufficient to induced Fas expression in parental HT29 cells as evaluated by flow cytometry (data not shown).

Because the cell death induced by IFN-λ signalling occurred relatively late (48 h; Fig. 1e), it is likely that gene products induced by IFN-λ may mediate apoptosis. Therefore, we determined whether short triggering of IFN-λ signalling would be sufficient for subsequent apoptosis or whether constitutive IFN-λ signalling should be maintained for the induction of apoptosis. To investigate this, HT29/FL-10R1/λR1 cells were treated with IL-10 to induce IFN-λ signalling, and the IL-10-containing media were replaced at different time points with fresh media to remove IL-10 and terminate IFN-λ signalling. The percentage of viable cells was measured at 48 h after addition of IL-10 (Fig. 3h). Removal of IL-10 resulted in marked time-dependent inhibition of cell death (Fig. 3h), demonstrating that constant presence of IFN-λ signalling was required for induction of cell death. These results suggest that either expression levels of some IFN-λ-induced gene products should reach a certain threshold to trigger apoptosis, and/or these IFN-λ-induced products should be further affected by IFN-λ signalling, to mediate apoptosis.

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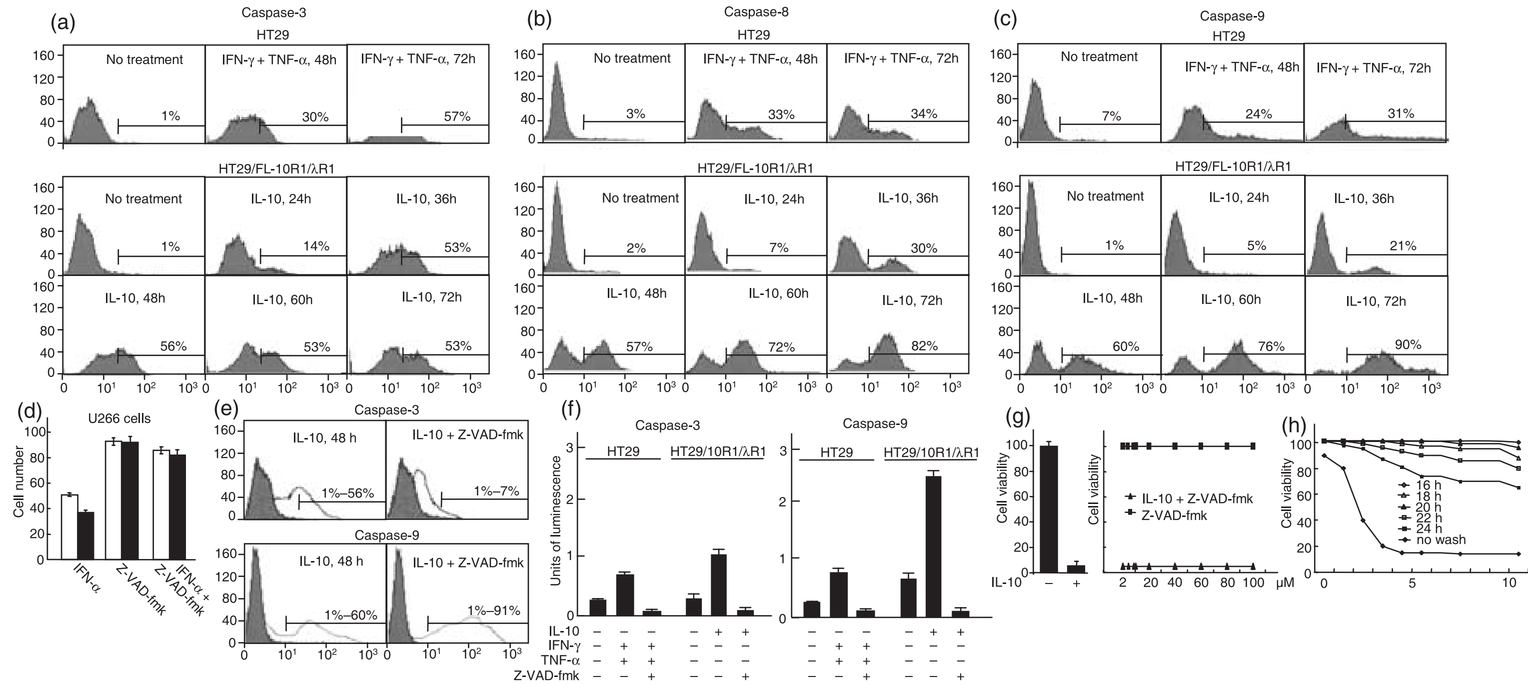

Figure 3. Activation of caspases. (a–c) Parental HT29 cells were left untreated or were treated with IFN-γ (10 ng/mL) and TNF-α (1 ng/mL) for 48 and 72 h (upper part of each panel) and HT29/FL-10R1/λR1 cells were left untreated or treated with IL-10 (10 ng/mL) for 24, 36, 48, 60 and 72 h (lower part of each panel), and activation of either caspase-3 (a), caspase-8 (b) or caspase-9 (c) was detected by flow cytometry with antibodies specific to activated caspases. (d) U266 cells were left untreated or treated with IFN-α (20 ng/mL), Z-VAD-FMK (30 μM) alone, or a combination of IFN-α and Z-VAD-FMK for 48 h (open bars), or 72 h (close bars). The ordinate represents cell number after the treatment shown as percentage to the control untreated cells. (e) Caspase-3 (top panel) and caspase-9 (bottom panel) activation was detected by flow cytometry after 48 h treatment with IL-10 alone (10 ng/mL) or a combination of IL-10 (10 ng/mL) and Z-VAD-FMK (30 μM) in HT29/FL-10R1/λR1 cells (open histograms). Untreated cells served as a control (shaded histograms). (f) Activities of caspases 3 and 9 in HT29 cells untreated or treated for 48 h with IFN-γ (10 ng/mL) and TNF-α (1 ng/mL) with or without Z-VAD-FMK, and in HT29/FL-10R1/λR1 cells untreated or treated for 48 h with IL-10 (10 ng/mL) with or without Z-VAD-FMK, were measured by luminescence caspase activity assay. Here and in Figs 5e and 6d, the ordinate represents the number of relative light units. (g) HT29/FL-10R1/λR1 cells were left untreated or treated with IL-10 (10 ng/mL) alone, or with increasing concentration of Z-VAD-FMK (2–100 μM) alone or in combination with IL-10 (10 ng/mL) for 48 h, and viability of the cells was determined. The abscissa represents the concentration of Z-VAD-FMK (μM), and the ordinate represents the percentage of live cells relative to the control untreated cells. (h) Viability of HT29/FL-10R1/λR1 cells was tested after 48 h of either continuous IL-10 (10 ng/mL) treatment, or when IL-10 treatment was discontinued, at the indicated time points, by replacing conditioned media with fresh media.

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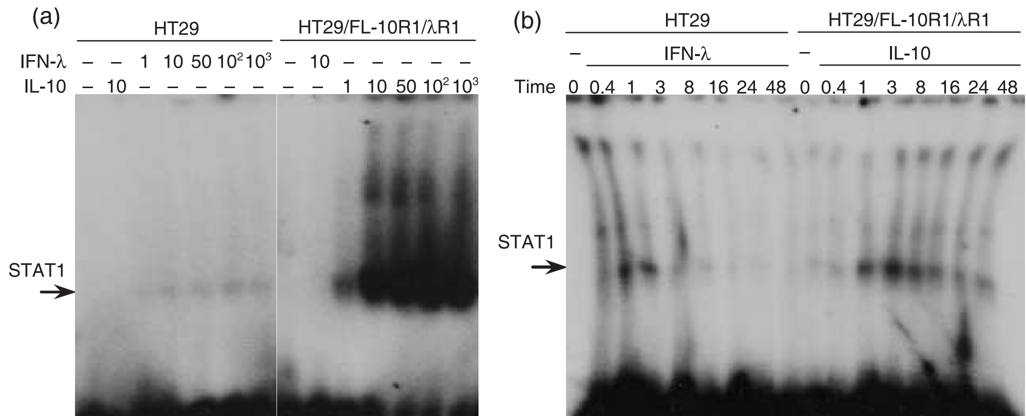

Figure 4. STAT1 activation in HT29 and HT29/FL-10R1/λR1 cells. (a) HT29 cells or HT29/FL-10R1/λR1 cells were left untreated or treated with IL-10 (10 ng/mL) or IFN-λ (10 ng/mL), or increasing concentrations of IFN-λ (1–1000 ng/mL) and IL-10 (1–1000 ng/mL), respectively, and STAT1 activation was measured by electrophoresis mobility shift assay. (b) HT29 cells or HT29/FL-10R1/λR1 cells were left untreated or treated with IFN-λ (100 ng/mL) or IL-10 (0.3 ng/mL), respectively, and STAT1 activation was determined in cells at various time points (h) of the treatment.

Apoptosis is affected by the strength of IFN-λ signalling

Although HT29 cells were responsive to all three types of IFNs (Fig. 1a), IFN-λ, even at the high concentration of 1 μg/mL, did not induce apoptosis in parental HT29 cells (data not shown). However, in HT29 cells expressing FL-10R1/λR1, IL-10 treatment induced IFN-λ signalling that lead to apoptosis (Figs 1–3), suggesting that signalling induced through the endogenous IFN-λ receptor complex and through the chimaeric IL-10 receptor complex may differ. Because IL-10 forms a dimer whereas IFN-λ is a monomer (Walter & Nagabhushan 1995; Zdanov *et al*. 1995; Kotenko *et al*. 2003), IL-10 dimers may induce stronger signalling than those induced by IFN-λ monomers. However, active IL-10 monomer (Josephson *et al*. 2000) was still fully capable of inducing apoptosis in HT29/FL-10R1/λR1 cells (data not shown), demonstrating that additional oligomerization of the receptor was not required for induction of apoptosis through the chimaeric 10R/λR receptor complex.

Signalling strength can also determine whether cells will undergo apoptosis. We were unable to overexpress intact IFN-λR1 chains in HT29 cells, suggesting that perhaps IFN-λ constitutively produced by the cells, may trigger IFN-λ signalling that leads to apoptosis in cells expressing high levels of the receptor. The expression level of endogenous IFN-λR1 chain limits intensity of IFN-λ signalling, and, therefore, even high amounts of exogenous IFN-λ can not induce apoptosis. Next, we compared strength of IFN-λ signalling induced in parental HT29 cells treated with IFN-λ with that induced by IL-10 in HT29/FL-10R1/λR1 cells. STAT1 activation was measured by EMSA. The results indicated that in response to the same levels of cytokine, IFN-λ signalling was much stronger in IL-10-treated HT29/FL-10R1/λR1 cells than in parental HT29 cells treated with IFN-λ (Fig. 4a). These results suggest that expression level of the receptors may determine whether apoptosis could be induced in the cells.

We also observed that IFN-α-, IFN-γ- and IFN-λ-induced signalling, and activities were attenuated in HT29/FL-10R1/λR1 in comparison with those in parental HT29 cells (data not shown). Because IFN receptors share many downstream signalling participants such as JAK kinases and STATs (Kotenko & Langer 2004; Pestka *et al*. 2004), it is likely that the FL-10R1/λR1

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chain competes with endogenous IFN receptor complexes for limited amount of signalling molecules, even in the absence of ligand binding.

Next, we determined whether the duration of IFN-λ signalling induced by IFN-λ in HT29 cells was different from that induced by IL-10 in HT29/FL-10R1/λR1 cells. HT29 cells were constantly treated with 100 ng/mL of IFN-λ and STAT1 activation was assessed by EMSA at various time points (Fig. 4b). HT29/FL-10R1/λR1 cells were constantly treated with 0.3 ng/mL of IL-10, the IL-10 concentration which induced the level of STAT1 activation in HT29/FL-10R1/λR1 cells similar to that induced by 100 ng/mL of IFN-λ in HT29 cells (Fig. 4a), and did not kill the cells (Fig. 2e). Cells were collected at the same time points of treatment and STAT1 activation was determined (Fig. 4b). Interestingly, it appeared that IFN-λ signalling was maintained for longer in IL-10-treated HT29/FL-10R1/λR1 cells than in HT29 cells treated with IFN-λ. These results suggest that IFN-λ signalling induced through the endogenous IFN-λ receptor complex was quickly suppressed, whereas signalling through the FL-10R1/λR1 chain was only slightly attenuated. Thus, prolonged signalling may determine whether IFN-λ could induce apoptosis.

IFN receptors possess intrinsic ability to induce cell death

Next, we decided to extend our study to other IFNs and IL-10-related cytokines (Fig. 5a). Four additional chimaeric receptors with an IL-10R1 extracellular domain and transmembrane and intracellular domains of either IFN-αR2c, IFN-γR1, IL-20R1 or IL-22R1, signalling competent subunits of IFN-α, IFN-γ, IL-22 and IL-26 receptor complexes (Kotenko 2002; Kotenko & Langer 2004), were created. IL-20R1 or IL-22R1 in combination with IL-10R2 assembles functional receptor complexes for IL-26 and IL-22, respectively (Hor *et al*. 2004; Xie *et al*. 2000; Kotenko *et al*. 2001; Sheikh *et al*. 2004). Although IFN-αR2c and IFN-γR1 interact with IFN-αR1 and IFN-γR2, respectively, to form type I and type II IFN receptor complexes (Kotenko & Langer 2004), IFN-αR2c and IFN-γR1 mediate signal transduction specificity of type I and type II IFNs and are responsible for STAT recruitment (Greenlund *et al*. 1994; Kotenko *et al*. 1996, 1999; Kotenko & Pestka 2000; Wagner *et al*. 2002). The chimaeric receptors were expressed in HT29 cells and clones, demonstrating similar levels of receptor expression were selected (Fig. 5b), treated with different concentrations of IL-10, and cell proliferation was assessed at 72 h (Fig. 5c). Interestingly, cells expressing chimaeric receptors with the intracellular domains of IFN receptors demonstrated antiproliferative effects in response to IL-10. In contrast, IL-10 treatment increased proliferation of cells expressing either FL-10R1/20R1 or FL-10R1/22R1. Externalization of PS in response to IL-10 in cells expressing various chimaeric receptors was also compared (Fig. 5d). Binding of annexin V was observed only in cells expressing chimaeric receptors with the intracellular domains of IFN receptors. Cells expressing FL-10R1/IL-22R1 or FL-10R1/IL-20R1 chains did not reveal any signs of cell death (Fig. 5c,d, and data not shown). Caspase-3 and caspase-9 were active in cells expressing either FL-10R1/λR1 or FL-10R1/αR2c at 48 h after IL-10 treatment, as measured by cleavage of the luminogenic substrate assay (Fig. 5e). Z-VAD-FMK blocked activity of both caspases (Fig. 5e). An antiproliferative effect, externalization of PS and caspase activation were the strongest in cells expressing FL-10R1/λR1 chain, demonstrating that the intrinsic ability of the IFN-λR1 intracellular domain to trigger apoptosis was stronger than those of IFN-αR2c and IFN-γR1 intracellular domains.

IFN-γ renders HT29 cells sensitive to IFN-λ-induced apoptosis

Next, we investigated whether other stimuli can make HT29 cells sensitive to IFN-λ-induced apoptosis. A combination of IFN-γ and either IFN-α or IFN-λ at high concentration (100 ng/mL) demonstrated a stronger antiproliferative effect on HT29 cells than IFN-γ alone, whereas treatment of HT29 cells with either IL-22 (100 ng/mL) or IFN-α (100 ng/mL) did not affect

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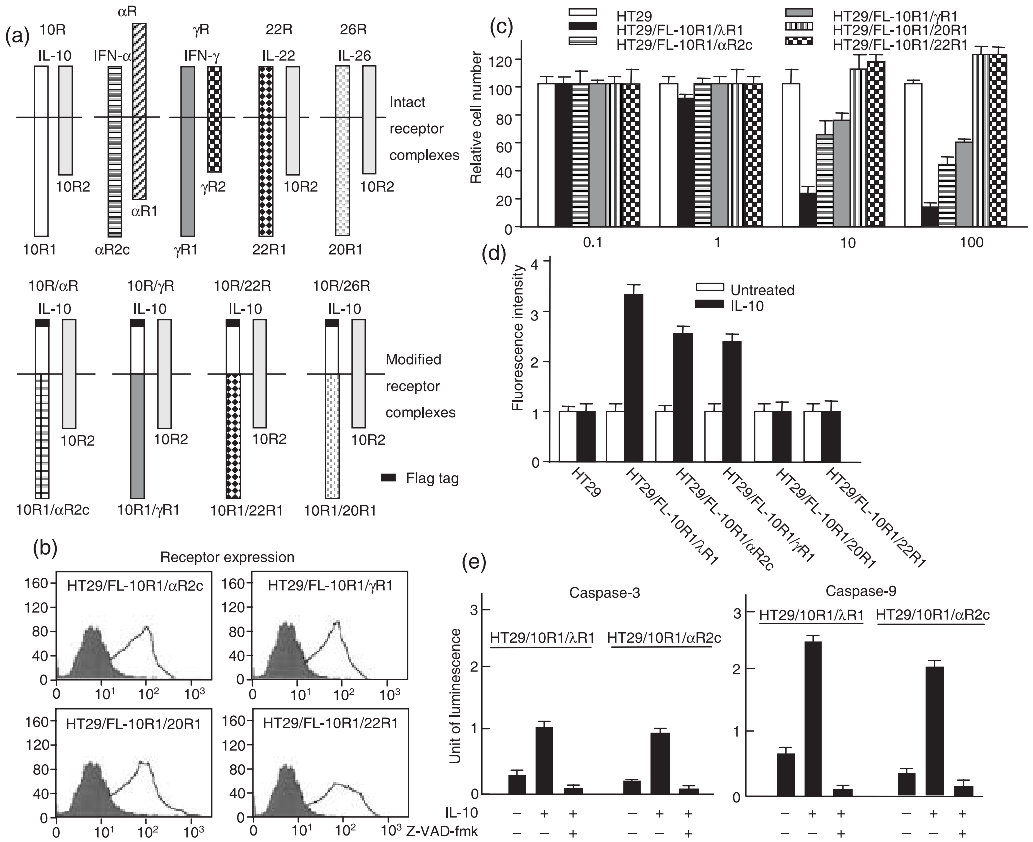

Figure 5. Signalling of all types of IFN induces apoptosis. (a) Receptors used in this study are shown schematically. Endogenous IL-10, IL-20, IL-22, IFN-α and IFN-γ receptor complexes (top panel) are each composed of a receptor chain with a short intracellular domain and a signalling-competent receptor chain with a long intracellular domain. Chimaeric receptor complexes (low panel) are each composed of the intact IL-10R2 chain and a chimaeric receptor subunit that has the IL-10R1 extracellular domain tagged with the FLAG epitope at the N-terminus, and either IFN-αR2c, IFN-γR1, IL-20R1 or IL-22R1 transmembrane and intracellular domains. (b) Expression of FL-10R1/αR2c, FL-10R1/γR1, FL-10R1/20R1, or FL-10R1/22R1 chain in clonal populations of HT29/FL-10R1/αR2c, HT29/FL-10R1/γR1, HT29/FL-10R1/20R1 or HT29/FL-10R1/22R1 cells (open histograms) in comparison to parental HT29 cells (shaded histograms) was tested by flow cytometry with FLAG antibody. (c) The cells were stimulated with various amounts of IL-10 for 72 h and their proliferation was evaluated by cell counting shown as a percentage of the control untreated cells. (d) Appearance of phosphatidylserine on the cell surface of the cells treated with IL-10 (100 ng/mL) for 48 h was measured by flow cytometry with FITC-conjugated annexin V. The ordinate represents a fold increase of the mean of fluorescence over the control untreated cells. (e) Activities of caspases 3 and 9 in HT29/FL-10R1/λR1 and HT29/FL-10R1/αR2c cells untreated or treated for 48 h with IL-10 (10 ng/mL) with or without Z-VAD-FMK were measured by luminescence caspase activity assay.

sensitivity of HT29 cells to IFN-λ (Fig. 6a). The ability of IFN-γ and IFN-λ co-treatment to inhibit proliferation was cell line-specific, as co-treatment had no effect on proliferation of colorectal adenocarcinoma COLO-205 or Caco-2 cell lines (data not shown). Next, we demonstrated that low concentration of IFN-γ, that alone had no or minimal effect on cell proliferation, PS externalization and caspase activation, still strongly increased sensitivity of HT29 cells to IFN-λ-induced apoptosis (Fig. 6b–d). IFN-γ potentiates the activity of type I IFNs by up-regulating expression levels of signalling molecules, such as STAT1, STAT2 and IRF9 (Improta *et al*. 1992;

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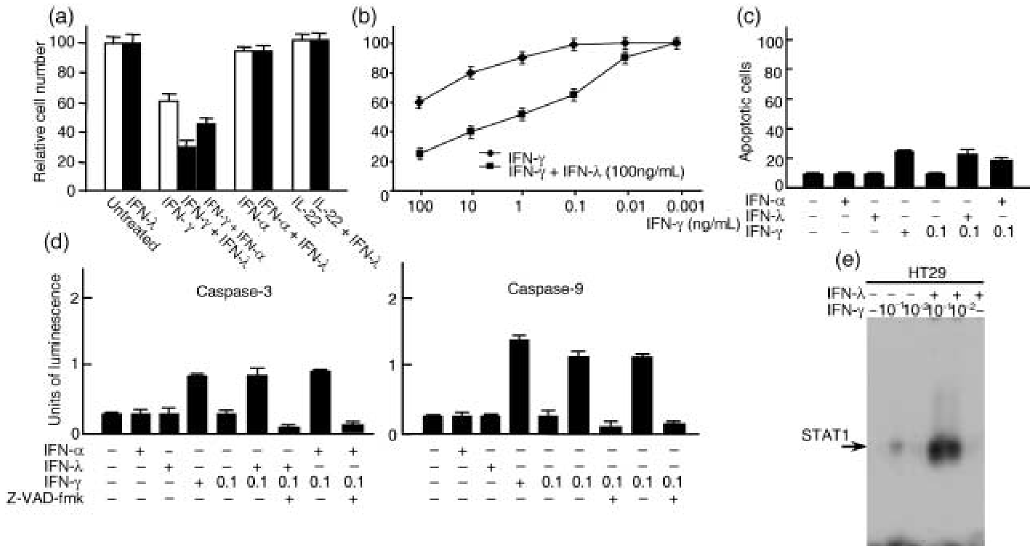

Figure 6. IFN-γ sensitizes HT29 cells to IFN-λ treatment. (a) HT29 cells were stimulated with IFN-λ (100 ng/mL), IFN-α (100 ng/mL), IFN-γ (100 ng/mL), or IL-22 (100 ng/mL), each cytokine alone or in combination, for 72 h, and cell proliferation was evaluated by cell counting shown as a percentage to the control untreated cells. (b) HT29 cells were treated with various amounts of IFN-γ alone or in combination either with IFN-λ (100 ng/mL) or IFN-α (100 ng/mL) for 72 h and cell proliferation was evaluated. (c) HT29 cells were left untreated or treated with IFN-α (100 ng/mL), IFN-λ (100 ng/mL), IFN-γ (100 or 0.1 ng/mL), or a combination of IFN-γ (0.1 ng/mL) and IFN-λ (100 ng/mL), stained with FITC-conjugated annexin V and PI, and percentage of apoptotic (annexin V-FITC-positive, PI-negative) cells was determined by flow cytometry. (d) HT29 cells were treated as in (c) with or without Z-VAD-FMK, and activities of caspases 3 and 9 were measured by luminescence caspase activity assay. (e) HT29 cells were pre-treated with IFN-γ (0.1 ng/mL or 0.01 ng/mL) for 24 h, were harvested and then either left untreated or incubated with IFN-λ (100 ng/mL) for 15 min and STAT1 activation was measured by electrophoresis mobility shift assay.

Levy *et al*. 1990; Lehtonen *et al*. 1997), the same molecules also participate in IFN-λ signalling (Kotenko *et al*. 2003). Indeed, in HT29 cells pre-treated with low doses of IFN-γ, IFN-λ-induced STAT activation was also greatly increased (Fig. 6e). These results demonstrate that IFN-λ, similar to IFN-α, is capable of inducing apoptosis in parental unmodified HT29 cells sensitized by IFN-γ treatment.

DISCUSSION

Interferon-α (type I IFN) is widely used clinically for treatment of various malignancies (Parmar & Platanias 2003; Borden 2005). However, molecular and cellular mechanisms mediating anti-tumour activity of IFNs are not fully understood (Thyrell *et al*. 2002; Chawla-Sarkar *et al*. 2003; Pokrovskaja *et al*. 2005; Maher *et al*. 2007). Type I IFNs exert their antitumour action by modulating numerous functions of immune cells and surrounding tumour stroma (Lindner 2002; Dunn *et al*. 2004, 2005). In addition, IFNs may also act directly on tumour cells inhibiting their proliferation and/or inducing apoptosis (Manabe *et al*. 1993; Sangfelt *et al*. 1997; Otsuki *et al*. 1998; Chen *et al*. 2001; Gamero *et al*. 2006b). Direct activation of apoptosis by IFNs can be observed only in selected cell types (Sangfelt *et al*. 1997; Otsuki *et al*. 1998; Luchetti *et al*.

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1998; Gamero *et al*. 2006a,b). Paradoxically, type I IFN can also promote proliferation or protect certain cells from apoptosis (Robert *et al*. 1986; Jewell *et al*. 1994; Pilling *et al*. 1999; Marrack *et al*. 1999; Ruuth *et al*. 2001; Gomez & Reich 2003), but the molecular mechanisms underlying such selectivity of type I IFN action remain undefined (Grander & Einhorn 1998; Chawla-Sarkar *et al*. 2003; Clemens 2003).

Recently identified type III IFNs (IFN-λs) act through a receptor complex that is distinct and independent of type I IFN receptor complex. Nevertheless, both types of IFN induce similar signal transduction events, ISGs and biological activities *in vitro* (Kotenko & Donnelly 2006; Kotenko 2007). Recently, antitumour potential of IFN-λs was also demonstrated in mouse models of tumour growth (Lasfar *et al*. 2006; Sato *et al*. 2006; Numasaki *et al*. 2007). Therefore, we aimed to investigate the ability of type III IFNs to induce apoptosis.

The expression pattern of IFN-λR1 is more restricted that those of type I IFN receptors, limiting IFN-λ activity to specific cell types (Lasfar *et al*. 2006; Doyle *et al*. 2006; Zhou *et al*. 2007). Accordingly, fewer cells respond to IFN-λ by antiproliferative action (Meager *et al*. 2005). Colorectal adenocarcinoma p53-deficient HT29 cells are often used as a model of intestinal epithelial cell differentiation and apoptosis (Wilson & Browning 2002). These cells are responsive to all three types of IFN (Kotenko *et al*. 2003; Fig. 1A). However, type I and type III IFNs failed to stimulate apoptosis in these cells (Figs 1 and 6). Ability of IFN-λ to induce antiproliferative effects correlated with the level of IFN-λR1 expression (Meager *et al*. 2005). To increase the level of IFN-λR1 expression, HT29 cells were transfected with expression plasmid encoding FL-IFN-λR1, but cells expressing detectable levels of FL-IFN-λR1 did not survive (data not shown). To investigate the intrinsic ability of IFN-λ signalling to regulate cell proliferation, we expressed the FL-10R1/λR1 chain in HT29 cells (Fig. 1d). Because IL-10 and IFN-λs share the second chain of their corresponding receptor complexes (Fig. 1c), HT29 cells expressing FL-10R1/λR1 chimaeric receptors respond to IL-10 by inducing IFN-λ signalling events (Kotenko *et al*. 2003). IL-10 treatment of HT29/FL-10R1/λR1 resulted in the induction of cell cycle arrest in G_0/G_1 phase (Fig. 2a). Morphological changes characteristic of apoptosis were observed, including gradual translocation of PS from the inner to the outer leaflet of the plasma membrane (Fig. 2b,c) and activation of caspase-3, -8 and -9 (Fig. 3a–c,f). At 48 h, DNA fragmentation was apparent (Fig. 2d), the cells became round and lost their adherence (Fig. 1e). Interestingly, apoptosis of HT29/FL-10R1/λR1 induced by IFN-λ signalling occurred relatively late (48 h; Fig. 1e) and was strongly dependent on continuous presence for 48 h of IL-10 in conditioned media, as removal of IL-10 even after 24 h of treatment markedly attenuated cell death (Fig. 3h). Therefore, it is likely that not only certain IFN-λ-induced gene products should be expressed but also should be triggered by IFN-λ signalling to mediate apoptosis. For example, IFNs up-regulate the cyclin-dependent protein kinase inhibitor p21, that promotes cell cycle arrest and suppresses apoptosis (Chin *et al*. 1996; Bissonnette & Hunting 1998; Hobeika *et al*. 1999; Seoane *et al*. 2002). However, when caspases are activated, p21 cleavage occurs; this promotes apoptosis of cancer cells (Zhang *et al*. 1999).

Type I IFN-mediated apoptosis in multiple myeloma U266 cells or in H123 cells (a variant of Jurkat T cells), occurs through the mitochondrial pathway, and pancaspase inhibitor Z-VAD-FMK was able to reduce, but not completely block apoptosis of the cells in response to IFN-α (Thyrell *et al*. 2002; Gamero *et al*. 2006a). In our experiments, Z-VAD-FMK did not block but even enhanced cleavage of caspase-9 in HT29/FL-10R1/λR1 cells undergoing IFN-λ signalling (Fig. 3e), demonstrating that caspase-9 cleavage may proceed through a caspase-independent mechanism. However, activity of caspase-9 was completely blocked by Z-VAD-FMK (Fig. 3f). It should also be noted that in addition to being cleaved, activation of caspase-9 requires active dimerization by an adaptor molecule (Green 2005). In contrast, cleavage and the activity of

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caspase-3 was blocked by Z-VAD-FMK (Fig. 3e,f), suggesting that caspase-3 activation is mediated by other caspases. Nevertheless, inhibition of caspase-3 activation was unable to prevent apoptosis in HT29/FL-10R1/λR1 cells undergoing IFN-λ signalling (Fig. 3g). Thus, IFN-λ-induced apoptosis can proceed through a caspase-independent pathway. Similar inability of Z-VAD-FMK to block apoptosis of HT29 cells induced in response to ligands of the TNF family has previously been described (Wilson & Browning 2002).

Although IFN-λs have been reported to induce antiproliferative responses in some cell lines (Brand *et al*. 2005; Meager *et al*. 2005; Zitzmann *et al*. 2006), proliferation of HT29 cells was not affected by IFN-λ signalling induced through its endogenous receptor complex (Fig. 1b), but was strongly inhibited by the same signalling induced through the exogenously introduced FL-10R1/λR1 receptor chain (Figs 1e and 5c). The ability of IFN-λ signalling to block proliferation and induce apoptosis correlated with the level of FL-10R1/λR1 receptor expression (Fig. 1d,e) and strength and duration of signalling (Fig. 4a,b). IL-10-induced STAT1 activation in HT29/FL-10R1/λR1 cells was much stronger and lasted longer than STAT1 activation induced by IFN-λ in HT29 cells (Fig. 4a,b). Interestingly, it has been reported that a STAT2 mutant that caused prolonged signalling and induction of IFN-stimulated genes, also rendered cells sensitive to IFN-α-induced apoptosis (Scarzello *et al*. 2007). IFN-induced JAK-STAT signalling is tightly controlled by several negative regulatory mechanisms including deactivation of signalling by cytoplasmic and nuclear tyrosine phosphatases, inhibition of receptor and JAK functions by induced suppressor of cytokine signalling (SOCS) proteins, and block of STAT DNA interaction by proteins that inhibit activated STATs (PIAS) (Levy & Darnell 2002). It is likely that strong IFN-λ signalling can override this control.

We have also demonstrated that in a similar setting, all IFN receptors efficiently mediate induction of apoptosis (Fig. 5). In contrast, engagement of IL-20R1 and IL-22R1 stimulated cell proliferation. IL-20R1 and IL-22R1 receptors together with IFN receptors belong to the same receptor family and in combination with the IL-10R2 chain assemble receptor complexes for IL-26 and IL-22, respectively (Kotenko 2002; Kotenko & Langer 2004; Pestka *et al*. 2004). IL-20R1 and IL-22R1 can also pair with IL-20R2 to form receptor complexes for IL-19, IL-20 and IL-24 (Blumberg *et al*. 2001; Dumoutier *et al*. 2001). Interestingly, both IL-22 and IL-20 transgenic mice die within days after birth, sharing similar skin abnormalities comparable to those observed in psoriasis, including keratinocyte hypoproliferation (Blumberg *et al*. 2001; Xu *et al*. 2003). Thus, ligands signalling through IL-20R1 and IL-22R1 can induce proliferation in responsive tissues. Therefore, only IFN receptors, and not receptors for IL-10-related cytokines, possess the unique intrinsic capability to trigger apoptosis. Interestingly, signalling through type III IFN receptor induced stronger antiproliferative and apoptotic activities than those induced through type I or type II receptors (Fig. 5c–e), suggesting stronger cytotoxic potential of type III IFNs.

We have also demonstrated that parental unmodified HT29 cells treated with low concentrations of IFN-γ, that alone had no or minimal effect on cell proliferation, became sensitive to IFN-λ-induced apoptosis (Fig. 6a–d). IFN-γ treatment also potentiated STAT activation induced by IFN-λ in HT29 cells (Fig. 6e). These results demonstrate that some stimuli may enhance sensitivity of cells to IFN-λ.

In conclusion, we have demonstrated that similar to type I and type II IFNs, signalling induced through the type III IFN (IFN-λ) receptor can induce apoptosis in cells. However, apoptotic capability of IFN-λ appears to depend on the strength of IFN-λ-induced signalling in cells. Thus, up-regulation of expression levels of IFN receptor and critical signalling molecules and/or inhibition of negative regulators of IFN signalling can render cells sensitive to IFN-λ-mediated apoptosis, and, therefore, should be explored to enhance cytotoxic potential of IFNs for the treatment of cancer.

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