Carcinogenesis vol.29 no.7 pp.1334–1342, 2008
doi:10.1093/carcin/bgn149
Advance Access publication June 19, 2008

Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-α-dependent mechanism

Alessio Paone[1], Donatella Starace[1], Roberta Galli[1], Fabrizio Padula[1], Paola De Cesaris[2], Antonio Filippini[1], Elio Ziparo[1] and Anna Riccioli[1,*]

[1]Department of Histology and Medical Embryology, Istituto Pasteur-Fondazione Cenci Bolognetti, ''Sapienza'' University of Rome, 00161 Rome, Italy and [2]Department of Experimental Medicine, University of L'Aquila, 67100 L'Aquila, Italy

[*]To whom correspondence should be addressed. Tel: +39 0649766582; Fax: +39 064462854;
Email: anna.riccioli@uniroma1.it

Toll-like receptors (TLRs) are known to play a key role in the innate immune system particularly in inflammatory response against invading pathogens. Recent reports strongly indicate that they play important roles in cancer cells. Prostate cancer represents one of the most common cancer for which no cure is available once metastatic and androgen refractory. Since TLR3 has been recently suggested as a possible therapeutic target in some cancer cell lines, we studied TLR3 expression and functionality in two human prostate cancer cell lines, LNCaP and PC3. We report that both cell lines express TLR3 and that the TLR3 agonist poly (I:C) activates mitogen-activated protein kinases and induces inhibition of proliferation as well as caspase-dependent apoptosis. By using pharmacological and genetic approaches, we demonstrate the involvement of TLR3 in poly (I:C)-induced effects. We also show that a novel interferon-independent pathway involving protein kinase C (PKC)-α activation, upstream of p38 and c-jun N-terminal kinase, is responsible for poly (I:C) pro-apoptotic effects on LNCaP cells. To our knowledge, this is the first report describing a role of PKC-α in poly (I:C)-mediated apoptosis. The comprehension of the mechanisms underlying TLR3-mediated apoptosis can contribute tools to develop new agonists useful for the treatment of prostate cancer.

Introduction

Pattern recognition receptors in host cells recognize widely expressed pathogen macromolecules to provide rapid recruitment of inflammatory cells to the site of infection thereby triggering a cascade of antimicrobial functions, collectively called 'innate immunity'. Consequently, the innate response to pathogens can be decisive in determining the nature and magnitude of 'adaptive immunity' (1).

The Toll-like receptor (TLR) family is one of the largest and best-studied families of pattern recognition receptors consisting, in humans, of 11 known members, which bind different bacterial or viral components. In the past, synthetic polymers, mostly poly (I:C) and poly (A:U), have been used with variable efficiency as adjuvants to treat cancer patients, with the aim of inducing an interferon (IFN)-mediated anticancer immune response. This therapeutic approach preceded the discovery of the specific receptor for these polymers, belonging to the TLR family. Moreover, recently, the transduction pathways and the molecular control of TLRs have been clarified, opening new insight into the potential use of vaccine-based strategies.

Abbreviations: 7-AAD, 7-amino-actinomycin D; AR, androgen receptor; BrdU, 5-bromo-2-deoxyuridine; dsRNA, double-strand RNA; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; IFN, interferon; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PCa, prostate carcinoma; PI, propidium iodide; PKC-α, protein kinase-α; TIR, Toll/IL1R; TLR, Toll-like receptor; TUNEL, Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; TLR3-DN, Toll-like receptor 3-dominant negative; WT, wild-type.

At present, TLR agonists and antagonists are being developed for the treatment of cancer and as adjuvants for potent new vaccines to prevent or treat cancer (2,3). Prostate cancer represents one of the most common cancer diagnosed in males in Western countries. Standard pharmacological therapy, consisting in withdrawal of androgens, does lead to regression of the disease, but this regression is often transient and there is no cure for prostate cancer once it has become metastatic and androgen refractory. Consequently, many efforts are being made to identify novel targets and mechanism-based agents useful for treatment of this disease (4). Interestingly, a link between TLR activity and prostate cancer is suggested not only by epidemiological data correlating chronic inflammation with increased cancer risk but also by genetic evidence. In fact, sequence variants in *Tlr* gene cluster (*Tlr6–Tlr1–Tlr10*) (5) and in *Tlr4* (6) in humans are related to increased risk of prostate cancer, clearly indicating a role of TLRs in its aetiology. Schulz *et al.* (7) recently reported that TLR3 is down-regulated in a subset of prostate cancer samples compared with benign tissues and such TLR3 down-regulation is associated with higher recurrence, suggesting that TLR3 expression might be useful for cancer prognosis.

Mounting evidence shows that the enhancement of innate and adaptive immunity represents the principal mechanism by which TLR stimulation produces antitumour activity. However, a direct pro-apoptotic effect of TLR agonists on $TLR3^+$ and $TLR9^+$ tumour cells has been recently reported (8–10). Poly (I:C) is a synthetic molecule able to mime the action of double-strand RNA (dsRNA) that represents either genomic or life cycle intermediate material of many viruses. dsRNA binding activates TLR3 (11) that utilizes the adaptor protein, TRIF, which binds members of the receptor-interacting protein family. This pathway leads to activation of mitogen-activated protein kinases (MAPKs), nuclear factor-kappa B (NF-κB) and interferon regulatory factor-3 (12) and to the induction of type I IFNs, among which IFN-β, a cytokine critical for the host defence against viral infections and for the induction of apoptosis in several cell types; cell death is indeed one of the mechanisms that permit hosts to limit virus invasion.

Prostate intraepithelial neoplasia and early invasive carcinomas are characterized by increase in proliferation rate compared with normal prostate epithelium, whereas advanced and/or metastatic prostate cancers display an ∼60% decrease in the rate of apoptosis. Dysregulation of various cell cycle regulatory genes, such as *p27*, *p16* and *cyclin D1*, is prevalent in prostate tumours (13,14). Altered cell cycle control is therefore likely to play a role in the progression of clinically localized disease, whereas deregulated apoptosis may be more important for advanced carcinoma (15). Based on this evidence, we have investigated the effect of the TLR3-specific ligand poly (I:C) on the proliferative and apoptotic rates of two different human prostate cancer cell lines, LNCaP and PC3. We found that poly (I:C) elicits inhibition of proliferation associated with a significant induction of TLR3-mediated apoptosis in both prostate cancer cell lines. In experiments aimed at shedding light into the mechanisms activated by poly (I:C), we identified a new IFN-β-independent pathway that involves protein kinase C (PKC)-α activation as responsible for the observed effects of poly (I:C) on LNCaP cell line.

Materials and methods

Cell lines

LNCaP and PC3 prostate cancer cells were maintained in Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin–streptomycin and 10% fetal calf serum (FCS) (Sigma–Aldrich, St Louis, MO). For signal transduction experiments, cells were serum starved for 24 h and then stimulated with poly (I:C) in FCS-free medium; otherwise, cells were treated in medium containing 3% FCS.

PC3 cells stably transfected with human full-length androgen receptor (AR) construct (p5HbhAR-NEO), containing the full-length human AR complementary

DNA, or vector alone (PC3–NEO cells) were kindly provided by Dr L.Bonaccorsi, University of Florence (16). Cells were cultured in the presence of 0.5 mg/ml geneticin (G418, Calbiochem–Merck).

Reagents

Poly (I:C) was from Invivogen (San Diego, CA), U0126 and SB203580 were from Calbiochem-Merck (Darmstadt, Germany), SP600125 was from Tocris Bioscience (Ellisville, MO), Gö6976 was from Calbiochem-Merck and Bafilomycin A1 was from Sigma (St. Louis, MO). Cell viability after exposure to all the inhibitors (at the highest concentrations used) was assessed by trypan blue exclusion.

Apoptosis assays

Propidium iodide (PI) staining: cells were detached with trypsin, washed with cold phosphate-buffered saline (PBS)–5% FCS and then fixed in 70% ethanol for 24 h. After washing with PBS, cells were incubated with 1 μg/ml PI for 3 h at 25°C before FACS analysis by Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Cells were considered apoptotic when their DNA content was $<2N$.

AnnexinV staining: Cells were detached with trypsin, washed with PBS–5% FCS and then placed in binding buffer containing 0.14 M NaCl, 2.5 mM $CaCl_2$ and 0.01 M *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (pH 7.4) to which 7-amino-actinomycin D (7-AAD) and annexin V-FITC (Pharmingen, San Diego, CA) were added prior to FACS analysis. Cells were considered apoptotic when annexin V-FITC positive and 7-AAD negative.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining was performed using 'In situ cell death detection kit, TMR red' (Roche, Basel, Switzerland), according to the manufacturer's instructions. Results presented as percentage of specific apoptosis were determined using the following formula: [(% apoptotic cells in experimental − % apoptotic cells in control)/(100 − % apoptotic cells in control) × 100]. The results from the apoptosis assays were analyzed with Win MDI software (Scripps Research Institute, La Jolla, CA).

Proliferation assay

The incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA of proliferating cells was measured by a FITC–BrdU flow kit (BD Pharmingen, San Diego, CA) according to the manufacturer's protocol. Cells were fixed, permeabilized and total DNA was stained with 7-AAD, followed by FACS analysis.

Sub-cellular fractionations

Following treatment with 100 μg/ml poly (I:C) or 0.2 μM 12-*O*-tetradecanoylphorbol-13-acetate (Sigma), cells after washing were scraped in 1 ml PBS and centrifuged (2000*g*) for 3 min at 4°C. Cell pellets were resuspended in hypotonic homogenization buffer (20 mM Tris, 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid and 5 mM dithiothreitol) and protease inhibitors, sonicated on ice (five 15 s pulses), incubated on ice for 15 min and ultracentrifuged (35000*g* for 30 min at 4°C). Supernatants were collected as cytoplasmic fractions. Pellets were resuspended in isovolumes of homogenization buffer plus 0.1% Triton X-100, incubated on ice for 30 min and centrifuged (20000*g* for 10 min at 4°C). Supernatants were collected as membrane fractions.

Western blotting

Cell lysates were prepared in cell lysis buffer (Cell Signalling, Danvers, MA). Protein concentration was determined by the micro bicinchoninic acid method (Pierce, Rockford, IL).

Equal amounts of proteins (40 μg) were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane saturated with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20. Membranes were incubated with primary antibody and subsequently with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Membranes were washed with Tris-buffered saline with 0.1% Tween-20 and developed using the chemiluminescence system (ECL Advance; Amersham Bioscience, Piscataway, NJ). Source of primary antibodies: anti-phospho-p38, phospho-extracellular signal-regulated kinase (ERK) and cleaved caspase-3 (Asp175) from Cell Signalling; anti-phospho-c-jun N-terminal kinase (JNK) from BioSource International (Camarillo, CA); anti-caspase-8 from Medical & Biological Laboratories (Co.Naka-Ku Nagoya, Japan); anti-PKC-α from BD Pharmingen; anti-c-Myc, p21, p27, cyclin A1, B1 and D1 from Santa Cruz Biotechnology (Santa Cruz, CA) and α-tubulin and β-actin antibodies from Sigma. Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (Bio-Rad, Hercules, CA).

Transfection

One day after plating (1.4×10^5 cells/ml), cells were transiently transfected with 1 μg wild-type (WT)-PKC-α or negative dominant 'kinase-dead' mutant version of PKC-α (K368R, shown as PKC-α-DN), both kindly provided by Dr Baier (University of Innsbruck) (17) and/or with 1 μg Toll-like receptor 3-dominant negative (TLR3-DN) (TLR3-ΔTIR), a Toll/IL1R (TIR)-less form of the TLR3 gene generated by deleting the Toll/IL1R (TIR) domain (450 bp) (pZERO-hTLR3, Invivogen). Plasmid containing WT human p53 gene was kindly provided by Dr Vogelstein (Johns Hopkins University, Baltimore, MD) (18). Cells were transfected for 5 h with lipofectamine LTX/plus (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Stimulation with 25 μg/ml of poly (I:C) was performed 18 h later.

Statistical analysis

Statistical differences were determined either by Student's *t*-test for paired samples or by one-way analysis of variance followed by Student's *t*-test with the Bonferroni's correction. $P < 0.05$ was considered significant. Densitometric analysis was performed using AIDA software.

Results

TLR3-mediated MAPK activation in LNCaP and PC3 cell lines

To investigate the effect of TLR3 stimulation on prostate carcinoma (PCa) cell lines, we first studied the basal expression of TLR3 in LNCaP and PC3. Figure 1A shows that both cell lines express TLR3 protein at similar levels, as assessed by western blotting performed on whole-cell lysates.

Once verified that LNCaP and PC3 express TLR3, we evaluated its functionality by assaying the degree of MAPK activation induced by the dsRNA synthetic analogue poly (I:C). Cells were treated for the indicated times with 25 μg/ml poly (I:C) and whole-cell lysates were subjected to western blotting to study the kinetics of MAPK phosphorylation. Figure 1B shows that p38 and JNK follow the same kinetics of stimulation in both LNCaP and PC3, being activated after 1 and 2 h stimulation. Poly (I:C) failed to activate ERK above basal levels in PC3 cells, whereas in LNCaP, the kinetics of ERK activation was similar to that of p38 and JNK.

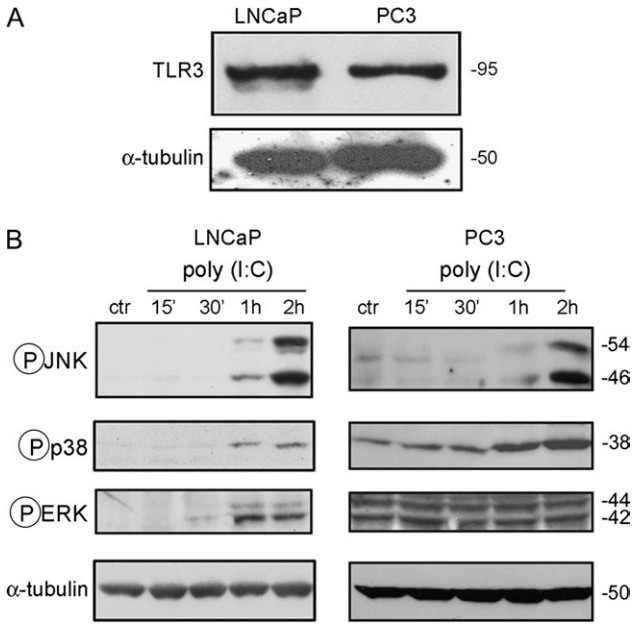

Fig. 1. Constitutive TLR3 expression and poly (I:C)-induced activation of MAPK in LNCaP and PC3 cells. (**A**) Western blot analysis of TLR3 expression in extracts obtained from the two PCa cell lines (40 μg/lane). (**B**) Cells were treated with 25 μg/ml of poly (I:C) for the indicated time range. Whole-cell lysates were subjected to western blot analysis for the phosphorylated forms of JNK, p38 and ERK. The same filters were reincubated with an anti-α-tubulin antibody as control for equal amount of protein loaded. Similar results were obtained in three independent experiments.

Poly (I:C) inhibits the proliferation of the two PCa cell lines

Since earlier studies have described that dsRNA exerts anti-proliferative effects in neoplastic cells (19), we investigated the effects of poly (I:C) on LNCaP and PC3 proliferation. The cells were stimulated for 18 h with 25 μg/ml poly (I:C) in medium containing 3% FCS and DNA synthesis was evaluated by BrdU incorporation. Figure 2A shows that in LNCaP cells, poly (I:C) treatment results in strong reduction (∼60%) of BrdU incorporation, suggesting that poly (I:C) significantly inhibits LNCaP proliferation. On the other hand, in PC3, poly (I:C) stimulation only slightly reduces BrdU incorporation (Figure 2A). Cell cycle progression is regulated by a series of cyclin-dependent kinases that, once activated, give rise to the dissociation and/or degradation of cyclin-dependent kinase inhibitors. We then determined whether cyclins are involved in poly (I:C)-mediated reduction of proliferation in both cell lines. Cells were stimulated for 6 and 18 h with poly (I:C) and whole-cell lysates were subjected to western blot analysis for specific cyclins. In LNCaP, cyclins B1 and A1 are not affected, whereas cyclin D1, a marker of G_1/S transition, is significantly reduced following 18 h poly (I:C) treatment (Figure 2B). In contrast, in PC3 cells, none of the cyclins tested is down-regulated by poly (I:C) (Figure 2B).

Since MYC plays a central role in cell proliferation and overexpression of MYC has been demonstrated in prostate cancer, we investigated whether MYC levels are affected by poly (I:C) treatment. Figure 2C shows that in LNCaP, but not in PC3, MYC degradation is induced starting after 6 h stimulation with poly (I:C), being fully accomplished after 18 h. We further analyzed the expression levels of various proteins involved in G_1/S-phase transition by investigating whether poly (I:C) treatment concomitantly stimulates cyclin-dependent kinase inhibitors p21 and p27. Our data show that both p21 and p27 are up-regulated by poly (I:C) treatment in LNCaP cells (Figure 2C). Moreover, in PC3 cells, poly (I:C) stimulation leads to a significant increase in p21 levels, whereas p27 levels are unaffected (Figure 2C). The densitometric values shown in Figure 2B and C indicate the difference of cell cycle protein expression in poly (I:C)-treated cells compared with the untreated cells.

Poly (I:C) induces caspase-dependent apoptosis of PCa cell lines

Prostate cancer cells are reported to be insensitive to several apoptotic stimuli (20). Recently, a direct apoptotic effect of poly (I:C) on breast cancer cells was demonstrated (9). To investigate whether poly (I:C) is able to induce apoptosis in LNCaP and PC3 cell lines, cells were treated for 48 h with poly (I:C), then stained with PI and subjected to cell cycle analysis by flow cytometry. PI staining for DNA content of LNCaP cells showed an apparent increase in sub-G_1 peak (apoptotic cells) up to ∼45% after 48 h poly (I:C) treatment (Figure 3A upper left panel). As regards PC3 cell line, Figure 3A (lower left panel) shows that the sub-G_1 population represents 12.6% of poly (I:C)-stimulated cells, indicating slight levels of apoptosis. To verify these results, we performed apoptosis analysis by using TUNEL assay in both PCa cell lines (Figure 3B) that confirmed the data obtained by PI staining. In order to investigate apoptosis in its early phase, LNCaP cells were treated for 24 h with poly (I:C) and then subjected to 7-AAD–annexin V-FITC staining. The histogram in Figure 3C shows that apoptotic cells are ∼2% in control samples, whereas they markedly increase up to 22% following poly (I:C) stimulation, which shows that induction of apoptosis in LNCaP cells is already detectable after 24 h poly (I:C) stimulation. 7-AAD–annexin V-FITC staining analysis was also performed in PC3 cell line and data obtained indicate that apoptotic cells represent 1.2% of PC3 control cells and poly (I:C) induces only a marginal but significant apoptotic response up to ∼9% (Figure 3C). To assess whether poly (I:C) stimulation induces activation of caspases, both PCa cell lines were treated with poly (I:C) for 18 h and analyzed by western blotting for activated forms of caspase-8 and caspase-3. Figure 3D shows that caspase-8 and caspase-3 are activated after poly (I:C) stimulation in both PCa cell lines, with caspase activation more efficient in LNCaP than in PC3 cells. These data show that the pro-apoptotic effect of poly (I:C)

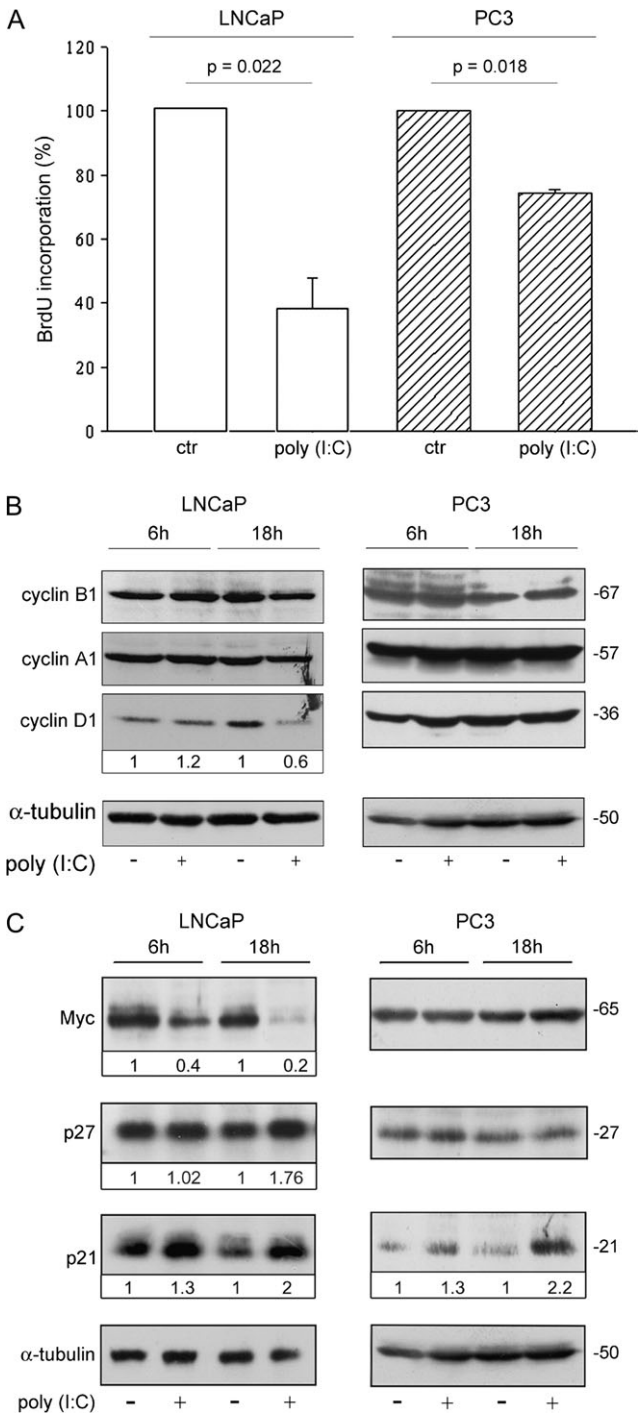

Fig. 2. Effects of poly (I:C) treatment on LNCap and PC3 cell cycle. (**A**) Incorporation of BrdU was evaluated in LNCaP and PC3 cells after treatment with 25 μg/ml poly (I:C) for 18 h, followed by addition of BrdU (10 μM) for 40 min. Details of the proliferation assay are described in the Materials and Methods. Results represent mean from three experiments with standard deviation as error bars. The exact *P*-values are reported versus respective controls, Student's paired *t*-test. (**B** and **C**) Western blot analysis of LNCaP and PC3 cells treated with poly (I:C) for the indicated times. The numbers below cyclin D1, c-Myc, p21 and p27 western blotting are the ratios between the densitometric values of treated versus control samples and represent the increase/decrease in induction levels. Data were normalized against α-tubulin density values. Data shown are from a typical experiment repeated three times with similar results.

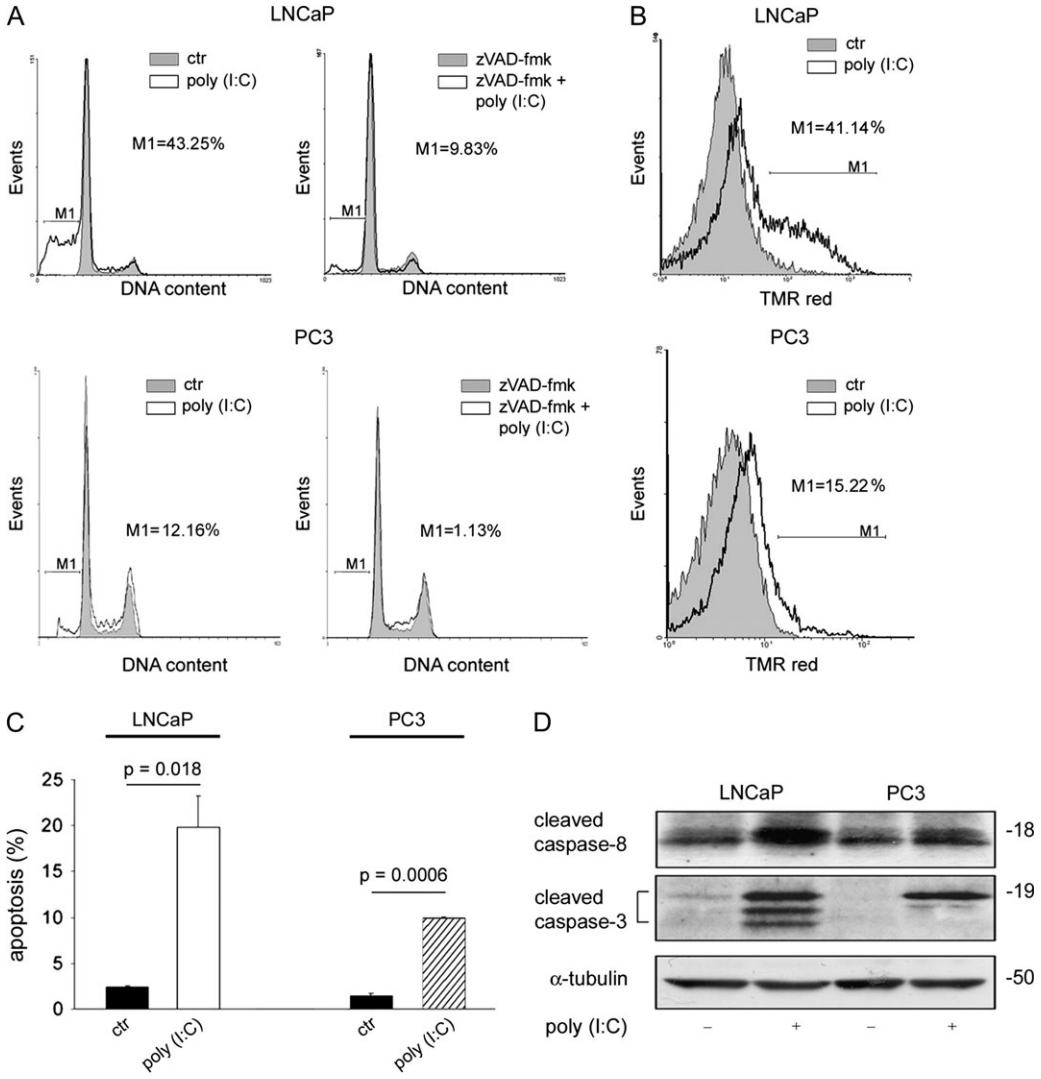

Fig. 3. Poly (I:C)-induced apoptosis in LNCaP and PC3 cells. (**A**, **B** and **C**) Flow cytometric analysis of apoptosis. (**A**) PI staining after 48 h stimulation with poly (I:C) alone or in combination with pan-caspase inhibitor zVAD-fmk (20 μM). (**B**) TUNEL assay performed after 48 h poly (I:C) stimulation. M1 marks the sub-G_1 cell fraction in (**A**) or the TUNEL-positive cells in (**B**) and corresponds to the percentage of apoptotic cells. (**C**) AnnexinV–7-AAD staining after 24 h treatment with poly (I:C). This histogram represents the mean ± SD of three independent experiments. The exact *P*-values are reported versus respective controls, Student's paired *t*-test. All the apoptosis assays are described in the Materials and Methods. (**D**) Western blotting for the activated forms of caspases-8 and -3 of both PCa cell lines, treated or not with poly (I:C); α-tubulin was used as loading control. Data in (**A**, **B** and **D**) represent typical experiments that were repeated three times with similar results.

treatment in LNCaP cells is much stronger than in PC3 cells. To determine whether caspase activation is involved in poly (I:C)-induced apoptosis, we treated both the PCa cell lines with the broad spectrum caspase inhibitor zVAD-fmk (20 μM) together with poly (I:C). Figure 3A (right panel) shows that zVAD-fmk greatly reverts the induction of apoptosis, with the sub-G_1 phase representing 9.8 and 1.13% in LNCaP and PC3, respectively, which suggests that poly (I:C)-induced apoptosis is caspase dependent. In addition, poly (I:C) appears to induce apoptosis in a bcl-2-independent fashion since its expression is unaffected by poly (I:C) treatment (data not shown).

Poly (I:C)-induced apoptosis in LNCaP and PC3 cells is TLR3 dependent

It has been demonstrated that endosomes contain TLR3 and that inhibitors of endosomal acidification abrogate TLR3-dependent poly (I:C) responses (21). To determine whether the signal transduction mechanisms activated by poly (I:C) in both LNCaP and PC3 cells depend on endosomal TLR3, cells were pre-treated for 1 h with the inhibitor of endosomal acidification bafilomycin A1 (100 nM) prior to 1 h poly (I:C) stimulation, then lysed and subjected to western blot analysis. Figure 4A shows that pre-treatment with bafilomycin A1 strongly inhibits poly (I:C)-induced JNK and p38 phosphorylation. These data suggest that poly (I:C)-activated signal transduction is dependent upon endosome-localized TLR3. To further study TLR3 involvement in poly (I:C)-induced apoptosis, we pre-treated LNCaP cells with bafilomycin A1 1 h prior to 24 h poly (I:C) stimulation. Cells were then assayed for 7-AAD–annexin V-FITC staining. Figure 4B shows that pre-treatment with bafilomycin A1 reduces apoptosis, with the annexin V-positive–7-AAD-negative cells representing 4.8 and 2.6% versus 17.6 and 10.1% found in poly (I:C)-stimulated LNCaP and PC3 cells, respectively. These data strongly suggest that poly (I:C)-induced apoptosis in both PCa cell lines is TLR3 dependent. To gain more insight into the direct role of TLR3 in poly (I:C)-dependent effects, both LNCaP and PC3 cells were transiently transfected either with a control plasmid or with a vector encoding a dominant-negative (DN) form of TLR3 (non-functional, due to deletion of the TIR domain) to compete with the endogenous functional TLR3. Transfected cells were treated with poly (I:C) for different

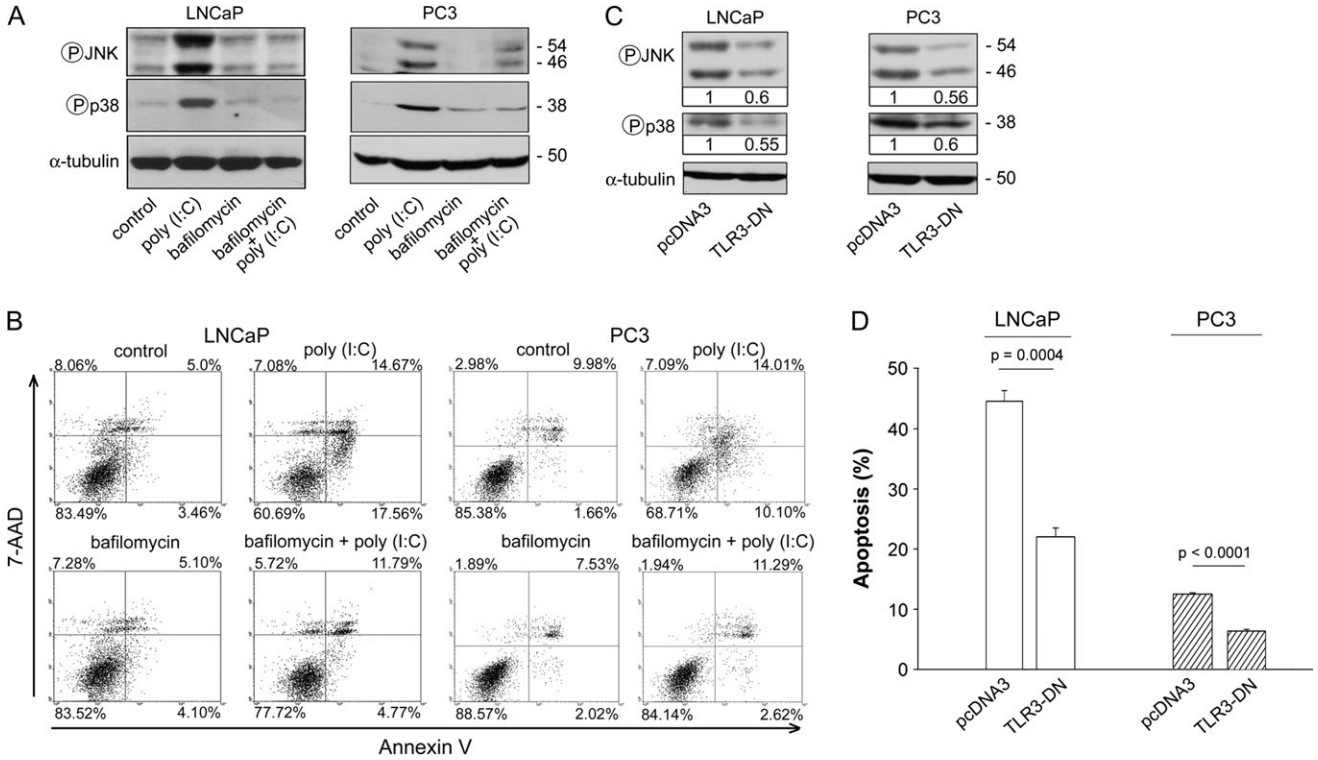

Fig. 4. Effects of TLR3 inhibition on poly (I:C)-induced responses. (**A**) Western blotting of both PCa cell lines untreated or poly (I:C) treated for 2 h with or without pre-treatment with the inhibitor of endosomal acidification bafilomycin A1 (100 nM). α-Tubulin content was used as loading control. (**B**) Apoptosis was evaluated by annexinV–7-AAD staining on LNCaP and PC3 cells after treatment with 25 μg/ml poly (I:C) with or without pre-treatment with bafilomycin A1. (**C** and **D**) pcDNA3 (control plasmid) or TLR3-DN was transiently introduced into LNCaP and PC3 cells. After 48 h, the cells were treated with poly (I:C) for 2 h prior to perform western blot analysis (**C**) and for 24 h prior to perform apoptosis evaluation by using PI staining (**D**). The numbers below JNK and p38 western blotting in (**C**) represent the values of densitometric analysis of the protein levels, calculated as the increase/decrease in induction by comparing TLR3-DN-transfected versus pcDNA3-transfected cells set arbitrarily at 1. Data were normalized against α-tubulin densitometric values. Data in **A**, **B** and **C** are from a typical experiment, repeated three times with similar results. In **D**, the histogram represents mean from three independent experiments with standard deviation as error bars. The exact *P*-values are reported versus PCa cells transfected with control plasmid, Student's paired *t*-test.

times and analyzed for p38 and JNK phosphorylation by western blotting and for apoptosis levels by PI staining. We found that down-regulation of TLR3 by TLR3-DN transfection significantly inhibits poly (I:C)-induced p38 and JNK phosphorylation ∼50% as assessed by densitometric analysis (Figure 4C). Accordingly, TLR3-DN over-expression reduces poly (I:C)-induced apoptosis to the same extent (Figure 4D). Overall, these data demonstrate a major role of TLR3 in poly (I:C)-induced responses in both PCa cell lines.

Role of over-expression of AR and p53 genes in poly (I:C)-induced apoptosis in PC3 cells

Since PC3 cells lack AR and p53 expression, the differences in response to poly (I:C) between LNCaP and PC3 cells might result from variations in the AR and p53 status of these cells. To determine whether these differences may account for the minor susceptibility of PC3 cells to poly (I:C)-induced effects, we performed apoptosis assay on PC3 cells stably transfected with AR plasmid or transiently transfected with p53 plasmid (PC3–p53) and treated with poly (I:C). Effectiveness of transfection was checked by western blot analysis (Figure 5A). As shown in Figure 5B, poly (I:C)-induced apoptosis was markedly increased in PC3–AR cells compared with PC3 cells transfected with control plasmid, whereas no increment was observed in PC3–p53 cells. Basal apoptotic levels were similar in all transfected PC3 cells (∼5–7%, data not shown). These results indicate that forced expression of AR in androgen-independent prostate cancer cell lines confers a higher sensitivity to poly (I:C)-induced apoptosis.

Since poly (I:C) induces ERK phosphorylation in LNCaP cells (Figure 1B), we investigated whether transfection of AR restores ERK sensitivity to poly (I:C) in PC3 cells. Figure 5C shows that ERK activation does not occur in poly (I:C)-treated PC3–AR. We conclude that in PC3–AR, similarly to LNCaP, ERK activation is not involved in the up-regulation of apoptosis induced by poly (I:C).

TLR3-mediated apoptosis in LNCaP cells is JNK and p38 dependent

Having observed that the effects of TLR3 stimulation are stronger in LNCaP than in PC3 cell line, we analyzed in more detail the apoptotic mechanisms in LNCaP cells. Since poly (I:C) induces MAPK activation (Figure 1B), we evaluated the effect of specific MAPK inhibitors on poly (I:C)-triggered apoptosis. To this aim, LNCaP cells were pre-treated with specific inhibitors for different times, depending on the inhibitor used, and then stimulated with poly (I:C) 48 h prior to apoptosis detection by PI staining. Results presented in Figure 6A show that pre-treatment with the ERK inhibitor U0126 (up to 10 μM) fails to inhibit apoptosis. An inhibitory effect was obtained with the JNK inhibitor SP600125 and an even more apparent effect with the p38 inhibitor SB203580 in a dose-dependent fashion. These data suggest that poly (I:C)-induced apoptosis is mainly p38 and JNK dependent. To further verify the involvement of p38 and JNK, we evaluated the effect of their specific inhibitors on caspase-8 and caspase-3 activation by western blot analysis. As expected, SB203580 and SP600125 were found to clearly inhibit poly (I:C)-induced caspase activation, whereas ERK inhibitor U0126 did not (Figure 6B).

PKC-α is upstream of JNK and p38 in regulating poly (I:C)-mediated apoptosis in LNCaP

It has been reported that 'classic' PKC activation mediates apoptotic response to phorbol esters in LNCaP cells (22) and that a potential

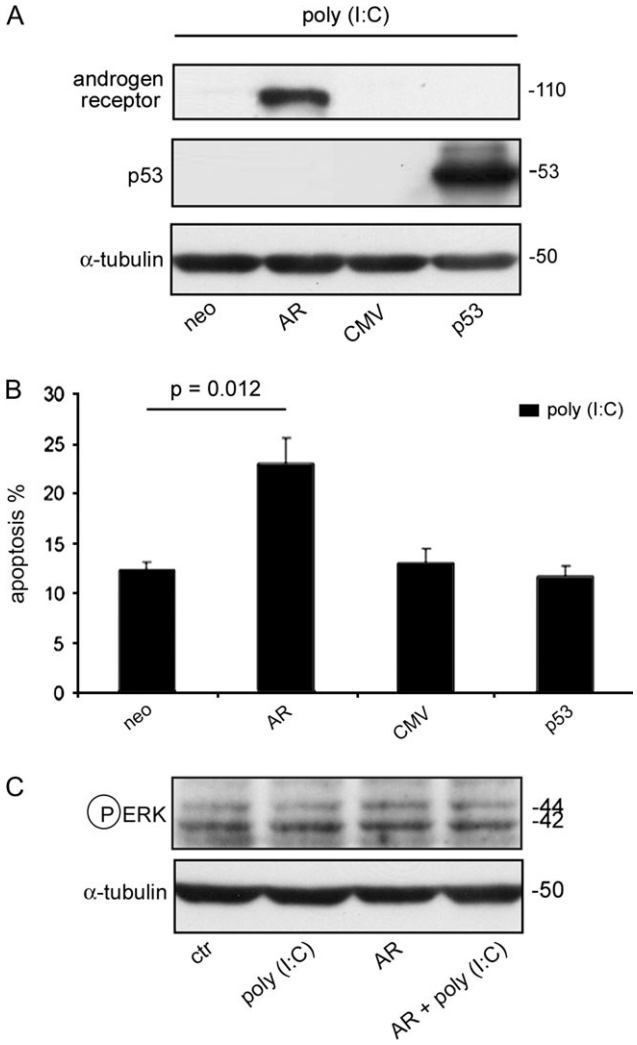

Fig. 5. Effect of p53 and AR over-expression on poly (I:C)-induced apoptosis in PC3 cells. (**A**) Control plasmid (CMV) or p53 expression vector (p53) was transiently introduced into PC3 cells; PC3–NEO (neo) and PC3–AR (AR) stably transfected cells were cultured in medium supplemented with 500 μg/ml geneticin. Cells were treated with poly (I:C) for 2 h prior to perform western blot analysis to detect p53 and AR protein expression. Data represent typical experiments that were repeated three times with similar results. (**B**) Transfected PC3 cells were treated for 24 h with poly (I:C) prior to perform apoptosis evaluation by using PI staining. Exact *P*-values are reported versus respective control, Student's paired *t*-test. (**C**) Parental PC3 [ctr and poly (I:C)] and PC3–AR stably transfected cells [AR and AR + poly (I:C)] were treated or not for 2 h with poly (I:C), and phosphorylation of ERK was examined as described in Materials and Methods.

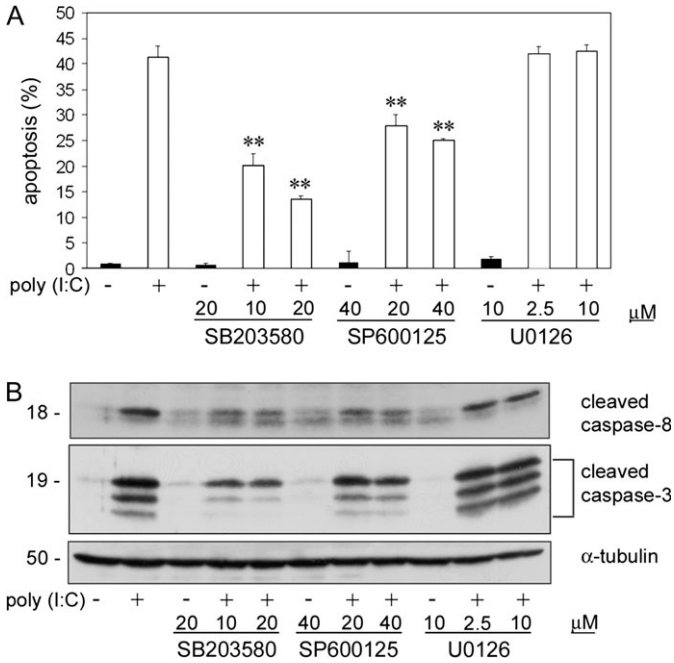

Fig. 6. Effects of MAPK inhibitors on poly (I:C)-mediated apoptosis. (**A**) PI staining was performed on LNCaP cells after 48 h of treatment with 25 μg/ml poly (I:C) alone or after pre-treatment with JNK inhibitor SP600125, p38 inhibitor SB203580 or ERK inhibitor U0126 for 1 h, 2 h and 5 min, respectively. Results represent the mean ± SD from three independent experiments. $^{**}P < 0.001$ versus poly (I:C) alone, Student's paired *t*-test. (**B**) Western blot analysis for the activated forms of caspases-8 and -3. U0126, SB203580 and SP600125 were added before poly (I:C) treatment. α-Tubulin was examined as loading control. Data are from a typical experiment repeated three times with similar results.

effector downstream of PKC is p38, but not JNK (23). Since PKC-α is reported to be the only classic PKC present in LNCaP cells (24), we investigated whether TLR3 stimulation triggers PKC-α activation in LNCaP. Figure 7A clearly shows PKC-α translocation to the plasma membrane after 1 h poly (I:C) treatment. Therefore, we analyzed whether PKC-α mediates the apoptotic effect of poly (I:C), by using both the specific pharmacological inhibitor Gö6976 (Figure 7C) and a dominant-negative PKC-α mutant (Figure 7B). Results obtained with both experimental approaches demonstrated that PKC-α is involved in TLR3-mediated apoptosis, as PKC-α down-regulation inhibits poly (I:C)-induced apoptosis assayed by PI staining (Figure 7B and C) and reverts caspase-3 cleavage in poly (I:C)-treated LNCaP (Figure 7D). To gain more insight into the role of PKC-α in poly (I:C)-induced apoptosis, a plasmid over-expressing WT-PKC-α was co-transfected together with TLR3-DN in LNCaP cells. The reduction in the percentage of apoptotic cells in WT-PKC-α/TLR3-DN-transfected cells versus control plasmid-transfected cells confirms that PKC-α activation by poly (I:C) depends on functional TLR3 (Figure 7B). Then, we studied the role of PKC-α in poly (I:C)-induced MAPK activation. As shown in Figure 7E, JNK and p38 activation is strongly diminished when PKC-α is blocked. Contrary to the effect observed on JNK and p38, PKC inhibitor Gö6976 does not affect ERK phosphorylation, indicating that PKC-α is upstream of the activation of JNK and p38, but not of ERK (Figure 7E).

Discussion

Among TLR members, only TLR2 and TLR9 have been described in human prostate cancer cell lines (25,26). Here, we report the expression of TLR3 in two prostate cancer cell lines, LNCaP and PC3, and its role in inhibiting cell cycle and inducing apoptosis. Currently, the potential of targeting TLRs in cancer is a much discussed issue since it became apparent that TLR expression is not restricted to innate immune cells but to be placed in tumour cells (27). Our data argue for an antitumour role of the TLR3 agonist poly (I:C) in prostate cancer in agreement with pioneristic reports showing that synthetic dsRNA polynucleotides can cause tumour regression (28). We studied the molecular mechanism underlying this reported beneficial effect of dsRNA and found that in PCa cells poly (I:C) reduces cell proliferation and increases apoptosis in a TLR3-dependent fashion. The involvement of TLR3 in the latter process was demonstrated by using an endosomal acidification inhibitor and by over-expressing a non-functional form of TLR3.

Our results indicate that poly (I:C) inhibits the proliferation of both LNCaP and PC3 cells, though at different extent. We demonstrate that in LNCaP cells poly (I:C) modulates key cell cycle regulatory proteins p21, p27, MYC and cyclin D1, whereas in PC3 cells only p21 is

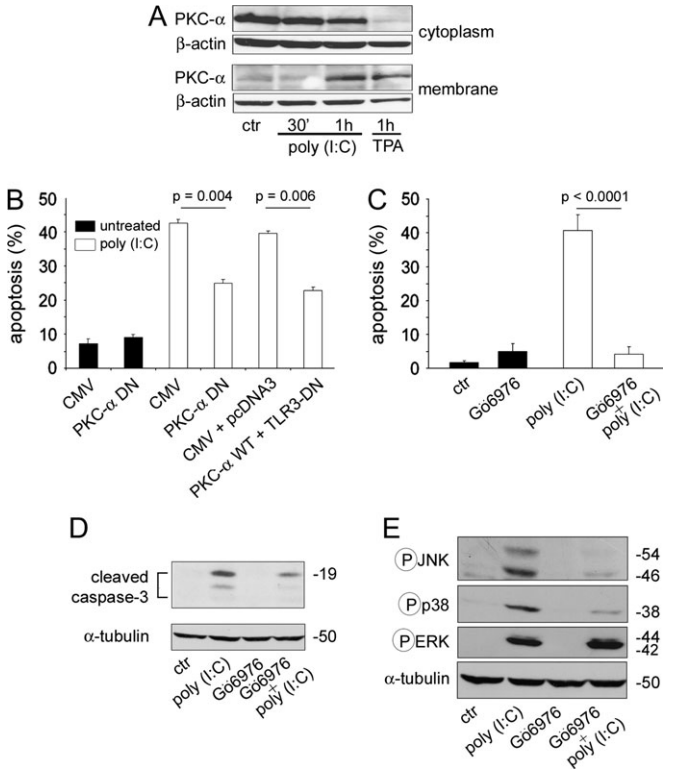

Fig. 7. Poly (I:C) effects observed in LNCaP are PKC-α dependent. (**A**) Western blotting for PKC-α was performed on cytoplasmic and membrane extracts from cells treated with poly (I:C) for the indicated times. For a positive control, cells were treated with the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA). β-Actin was examined as loading control. (**B**) PI staining of LNCaP cells transfected with a PKC-α dominant-negative (PKC-α-DN) plasmid or with control plasmid (CMV) treated or not with poly (I:C). Doubled-transfected cells (WT-PKC-α/TLR3-DN or respective control plasmids) were then treated with poly (I:C) for 24 h prior to PI staining for apoptosis evaluation. All the transfections were performed as described in Materials and Methods. (**C**, **D** and **E**) Cells were pre-treated or not with the inhibitor of PKC-α activation Gö6976 (0.2 μM), then treated with poly (I:C) either for 48 h prior to PI staining (**C**) or for 18 h prior to western blotting for caspase-3 (**D**) or for 2 h prior to western blotting for the phosphorylated forms of JNK, p38 and ERK (**E**). α-Tubulin was examined as loading control. In (**B** and **C**), the histograms represent mean from three independent experiments with standard deviation as error bars. The exact *P*-values are reported versus respective controls, Student's paired *t*-test (**C**); analysis of variance followed by Student's *t*-test with the Bonferroni's correction (**B**). In **A**, **D** and **E** data shown are from a typical experiment repeated three times with similar results.

affected and cell proliferation is inhibited much less than in LNCaP. The observed increase in cell cycle inhibitor p27 induced by poly (I:C) is in agreement with published data showing that in dendritic cells poly (I:C) treatment triggers p27-induced growth arrest (29). It is known that proliferating cells express higher levels of MYC and that repression of the cell cycle inhibitory gene p21 can contribute to the oncogenic properties of MYC (30). Here, we demonstrate that poly (I:C) induces both p21 up-regulation and MYC down-regulation, suggesting that the decrease in cell proliferation induced by poly (I:C) in LNCaP might be explained by MYC decrease resulting in p21 over-expression.

As regards poly (I:C)-induced apoptosis, the apoptotic rate of LNCaP cells following poly (I:C) stimulation is higher than that of PC3 cells, in accordance with the different degree of caspases-8 and -3 activation and with lack of ERK phosphorylation in PC3 cells versus LNCaP cells, suggesting that different mechanisms specific for genetic background regulate poly (I:C)-dependent apoptosis. Among differently expressed genes, WT p53 and AR are expressed in LNCaP cells, whereas PC3 cells, a more aggressive cell line, are null for p53 and AR. The dependence of prostate tumour on AR activity is exploited in treatment of disseminated prostate cancers, wherein ablation of AR function induces the regression of prostate tumours mainly through increased apoptosis. Tumour progression is associated with inappropriately restored AR function, despite sustained androgen ablation and/or the use of AR antagonists (31). Paradoxically, in normal prostate epithelium, a tumour suppressor function of AR has been demonstrated, but it is lost in androgen depletion-independent prostate cancers. However, PC3 cells retain the coregulators needed for AR tumour suppressor ability that can be restored following AR transfection (32). It is tempting to speculate that TLR3 pathway activation can somehow affect the complex AR-dependent regulation of cell cycle. In fact, poly (I:C)-induced apoptosis was increased in PC3–AR compared with WT null-AR PC3, indicating a correlation between AR expression and prostate tumour sensitivity to poly (I:C). Detailed comprehension of this intriguing issue certainly requires further investigation.

It is well known that p53 is a regulator of apoptosis in prostate cancer (33). Accordingly, WT p53-expressing LNCaP cells are more sensitive to selenite-induced apoptosis than p53-null PC3 cells and restoration of WT p53 expression in PC3 cells increases cellular sensitivity to selenite and results in increased apoptosis (34). Interestingly, our data demonstrate that transfection of WT p53 does not affect PC3 cell sensitivity to poly (I:C)-induced apoptosis.

As for NF-κB, it has been reported to be constitutively activated in androgen-independent PCa cell lines, such as PC3, in which NF-κB inhibition leads either to apoptosis or to sensitization to apoptosis induced by different treatments (35).

So far, a number of authors reported a strong pro-apoptotic effect of poly (I:C) only when combined to treatment with either cycloheximide or IFN-α (10,36,37). Surprisingly, we demonstrate a direct, considerable and TLR3-dependent pro-apoptotic effect of poly (I:C) in LNCaP cells. Accordingly, the involvement of TLR3 in dsRNA-triggered apoptosis of breast cancer cells has been described, though partially IFN dependent (9). Our data indicate that TLR3-mediated apoptosis can be IFN independent since LNCaP cells are IFN insensitive (38). Thus, alternative mechanisms must be operating in these cells to induce poly (I:C)-mediated apoptosis. Our data show that in LNCaP, dsRNA-induced apoptosis is mediated by caspase-8 and caspase-3. Accordingly, apoptosis induced by dsRNA in cells lacking IFN response was shown to be caspases-8 and -3 dependent (39).

We show for the first time that in cancer cell lines, poly (I:C)-induced apoptosis is mediated by PKC-α through the activation of its downstream effectors p38 and JNK, but not of ERK. The role of PKC-α and JNK in the initiation of apoptosis has been demonstrated in different cell types (40). A role for p38 has been demonstrated also in TLR2-dependent apoptosis (41) and reported in phorbol esters-induced PKC-mediated apoptosis in LNCaP cells, suggesting an important role of PKC-α and p38 in the growth control of this cell line (23). Khvalevsky *et al.* (42) described that signalling by TLR3 leads to the induction of apoptosis in the human hepatoma cell line HepG2 through receptor-interacting protein 3, a member of the Ser/Thr kinase family. Nevertheless, so far, a role of PKC-α has been demonstrated only as a component of TLR3 signalling regulating IFN-β gene expression (43). Our data therefore disclose a novel function for PKC-α in TLR3-dependent apoptosis. At present, it is unclear how PKC-α is involved in TLR3 signalling. The findings reported by Ivison *et al.* (44) might be helpful to elucidate this mechanism. These authors demonstrated that PKC-μ, a target of PKC-α, interacts with the TIR domain of TLR5, contributing to p38 activation. Moreover, they describe some non-specific interaction between TLR2 and PKC-μ. Interestingly, also another isoform of PKC, PKC-ε, directly interacts with a transducer protein associated to the TLR4 signalling pathway (45). According to these results, we may hypothesize that poly (I:C) binding to TLR3 might induce or enhance the interaction of TLR3 cytoplasmic TIR domain with cytosolic PKC-α that would then be able to activate downstream JNK and p38 in order to start the apoptotic process.

Finally, recent conflicting data indicate that, depending on the cell/system, innate immunity together with inflammation can either stimulate or inhibit tumour growth and progression. Ligands for TLR7 and TLR9 protect multiple myeloma from apoptosis (46). Prostate epithelial cell line proliferation increases in response to a number of TLR agonists common in the genitourinary system, suggesting a pathogenic link between prostate infection, inflammation and the development of PCa (47). Moreover, TLR9 has been detected also in breast and brain and TLR9 agonists stimulate metastatic invasion of prostate cancer cells (25,48). Our present data on prostate cancer cells contradict the general assumption of a tumour-promoting role of TLR, rather showing that, at least one of them, TLR3, might be directly involved in the down-regulation of PCa growth. To sum up, our data indicate that, following poly (I:C) stimulation, TLR3 signals to PKC-α that subsequently activates JNK and p38, leading to caspase-8-dependent apoptosis. Although several steps along the pro-apoptotic signalling pathway induced by TLR3 must still be elucidated, our data may provide a novel mechanism involved in TLR3-dependent apoptosis that could have great potential in new therapeutic approaches for anticancer strategies.

Funding

MIUR PRIN 2007; Agenzia Spaziale Italiana (ASI I/061/06/0).

Acknowledgements

We gratefully thank Fioretta Palombi for helpful discussion and critical and careful reading. We thank Dr L Bonaccorsi for providing the stably transfected PC3–AR cells and PC3–NEO cells.

Conflict of Interest Statement: None declared.

References

1. Akira,S. *et al*. (2006) Pathogen recognition and innate immunity. *Cell*, **124**, 783–801.
2. Kanzler,H. *et al*. (2007) Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat. Med.*, **13**, 552–559.
3. Krieg,A.M. (2008) Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. *Oncogene*, **27**, 161–167.
4. Fleshner,N. *et al*. (2007) Prostate cancer prevention: past, present, and future. *Cancer*, **110**, 1889–1899.
5. Sun,J. *et al*. (2005) Sequence variants in Toll-like receptor gene cluster (TLR6-TLR1-TLR10) and prostate cancer risk. *J. Natl Cancer Inst.*, **97**, 525–532.
6. Chen,Y.C. *et al*. (2005) Sequence variants of Toll-like receptor 4 and susceptibility to prostate cancer. *Cancer Res.*, **65**, 11771–11778.
7. Schulz,W.A. *et al*. (2007) Factor interaction analysis for chromosome 8 and DNA methylation alterations highlights innate immune response suppression and cytoskeletal changes in prostate cancer. *Mol. Cancer*, **6**, 14.
8. Jahrsdorfer,B. *et al*. (2005) Immunostimulatory oligodeoxynucleotides induce apoptosis of B cell chronic lymphocytic leukemia cells. *J. Leukoc. Biol.*, **77**, 378–387.
9. Salaun,B. *et al*. (2006) TLR3 can directly trigger apoptosis in human cancer cells. *J. Immunol.*, **176**, 4894–4901.
10. Salaun,B. *et al*. (2007) Toll-like receptor 3 expressed by melanoma cells as a target for therapy? *Clin. Cancer Res.*, **13**, 4565–4574.
11. Alexopoulou,L. *et al*. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, **413**, 732–738.
12. Kawai,T. *et al*. (2006) Innate immune recognition of viral infection. *Nat. Immunol.*, **7**, 131–137.
13. Drobnjak,M. *et al*. (2000) Overexpression of cyclin D1 is associated with metastatic prostate cancer to bone. *Clin. Cancer Res.*, **6**, 1891–1895.
14. Kibel,A.S. *et al*. (1998) Identification of 12p as a region of frequent deletion in advanced prostate cancer. *Cancer Res.*, **58**, 5652–5655.
15. Abate-Shen,C. *et al*. (2000) Molecular genetics of prostate cancer. *Genes Dev.*, **14**, 2410–2434.
16. Bonaccorsi,L. *et al*. (2000) Androgen receptor expression in prostate carcinoma cells suppresses alpha6beta4 integrin-mediated invasive phenotype. *Endocrinology*, **141**, 3172–3182.
17. Baier-Bitterlich,G. *et al*. (1996) Protein kinase C-theta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol. Cell. Biol.*, **16**, 1842–1850.
18. Baker,S.J. *et al*. (1990) Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science*, **249**, 912–915.
19. Lin,S.L. *et al*. (1982) Sensitivity and resistance of human tumor cells to interferon and rIn. rCn. *Nature*, **297**, 417–419.
20. Nesterov,A. *et al*. (2001) Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. *J. Biol. Chem.*, **276**, 10767–10774.
21. Niimi,K. *et al*. (2007) TLR3-mediated synthesis and release of eotaxin-1/CCL11 from human bronchial smooth muscle cells stimulated with double-stranded RNA. *J. Immunol.*, **178**, 489–495.
22. Garcia-Bermejo,M.L. *et al*. (2002) Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKCalpha. *J. Biol. Chem.*, **277**, 645–655.
23. Tanaka,Y. *et al*. (2003) Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. *J. Biol. Chem.*, **278**, 33753–33762.
24. Powell,C.T. *et al*. (1996) Persistent membrane translocation of protein kinase C alpha during 12-0-tetradecanoylphorbol-13-acetate-induced apoptosis of LNCaP human prostate cancer cells. *Cell Growth Differ.*, **7**, 419–428.
25. Ilvesaro,J.M. *et al*. (2007) Toll like receptor-9 agonists stimulate prostate cancer invasion *in vitro*. *Prostate*, **67**, 774–781.
26. Takeyama,K. *et al*. (2006) Prostate cell lines secrete IL-8 in response to Mycoplasma hominis through Toll-like receptor 2-mediated mechanism. *Prostate*, **66**, 386–391.
27. O'Neill,L.A. (2008) Toll-like receptors in cancer. *Oncogene*, **27**, 158–160.
28. Levy,H.B. *et al*. (1969) Inhibition of tumor growth by polyinosinic-polycytidylic acid. *Proc. Natl Acad. Sci. USA*, **62**, 357–361.
29. Hasan,U.A. *et al*. (2007) Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs. *Proc. Natl Acad. Sci. USA*, **104**, 8047–8052.
30. Claassen,G.F. *et al*. (2000) A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta-induced cell-cycle arrest. *Proc. Natl Acad. Sci. USA*, **97**, 9498–9503.
31. Hsieh,A.C. *et al*. (2007) Androgen-response elements in hormone-refractory prostate cancer: implications for treatment development. *Lancet Oncol.*, **8**, 933–939.
32. Litvinov,I.V. *et al*. (2006) PC3, but not DU145, human prostate cancer cells retain the coregulators required for tumor suppressor ability of androgen receptor. *Prostate*, **66**, 1329–1338.
33. Kirsch,D.G. *et al*. (1998) Tumor-suppressor p53: implications for tumor development and prognosis. *J. Clin. Oncol.*, **16**, 3158–3168.
34. Zhao,R. *et al*. (2006) Expression of p53 enhances selenite-induced superoxide production and apoptosis in human prostate cancer cells. *Cancer Res.*, **66**, 2296–2304.
35. Muenchen,H.J. *et al*. (2000) Tumor necrosis factor-alpha-induced apoptosis in prostate cancer cells through inhibition of nuclear factor-kappaB by an IkappaBalpha ''super-repressor''. *Clin. Cancer Res.*, **6**, 1969–1977.
36. Jiang,Q. *et al*. (2008) Poly I:C enhances cycloheximide-induced apoptosis of tumor cells through TLR3 pathway. *BMC Cancer*, **8**, 12.
37. Morikawa,T. *et al*. (2007) Identification of Toll-like receptor 3 as a potential therapeutic target in clear cell renal cell carcinoma. *Clin. Cancer Res.*, **13**, 5703–5709.
38. Dunn,G.P. *et al*. (2005) IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression. *Cancer Res.*, **65**, 3447–3453.
39. Weaver,B.K. *et al*. (2001) Apoptosis is promoted by the dsRNA-activated factor (DRAF1) during viral infection independent of the action of interferon or p53. *FASEB J.*, **15**, 501–515.
40. Reyland,M.E. *et al*. (2000) Activation of PKC is sufficient to induce an apoptotic program in salivary gland acinar cells. *Cell Death Differ.*, **7**, 1200–1209.
41. Into,T. *et al*. (2005) Apoptosis signal-regulating kinase 1-mediated sustained p38 mitogen-activated protein kinase activation regulates mycoplasmal lipoprotein- and staphylococcal peptidoglycan-triggered Toll-like receptor 2 signalling pathways. *Cell Microbiol.*, **7**, 1305–1317.
42. Khvalevsky,E. *et al*. (2007) TLR3 signaling in a hepatoma cell line is skewed towards apoptosis. *J. Cell. Biochem.*, **100**, 1301–1312.
43. Johnson,J. *et al*. (2007) Protein kinase Calpha is involved in interferon regulatory factor 3 activation and type I interferon-beta synthesis. *J. Biol. Chem.*, **282**, 15022–15032.

44. Ivison,S.M. *et al*. (2007) Protein kinase D interaction with TLR5 is required for inflammatory signaling in response to bacterial flagellin. *J. Immunol.*, **178**, 5735–5743.
45. McGettrick,A.F. *et al*. (2006) Trif-related adapter molecule is phosphorylated by PKC{epsilon} during Toll-like receptor 4 signaling. *Proc. Natl Acad. Sci. USA*, **103**, 9196–9201.
46. Jego,G. *et al*. (2006) Pathogen-associated molecular patterns are growth and survival factors for human myeloma cells through Toll-like receptors. *Leukemia*, **20**, 1130–1137.
47. Kundu,S.D. *et al*. (2008) The toll-like receptor pathway: a novel mechanism of infection-induced carcinogenesis of prostate epithelial cells. *Prostate*, **68**, 223–229.
48. Merrell,M.A. *et al*. (2006) Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity. *Mol. Cancer Res.*, **4**, 437–447.

Received February 20, 2008; revised May 28, 2008; accepted June 13, 2008