Toll-like Receptor 3 Expressed by Melanoma Cells as a Target for Therapy?

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Abstract

Purpose: The immunomodulatory properties of Toll-like receptors (TLR) agonists have inspired their use as experimental adjuvants for vaccination of cancer patients. However, it is now well recognized that TLR expression is not restricted to immune cells but can also be found in many cell types, including those giving rise to tumors. It is therefore mandatory to explore the potential effects of TLR triggering directly on tumor cells.

Experimental Design: In the present work, we have investigated TLR3 protein expression in melanoma cell lines derived from patients, and analyzed the effects of TLR3 agonists on tumor cell survival. Moreover, we used RNA interference to stably knock down TLR3 expression and study the involvement of this receptor in dsRNA-induced effects on melanoma cell viability.

Results: Human melanoma cells can express functional TLR3 protein. Interestingly, the engagement of the receptor by TLR3 agonists can directly inhibit cell proliferation and induce tumor cell death when combined to treatment with either type I IFN or protein synthesis inhibitors. These effects were shown by RNA interference to be largely dependent on TLR3. Moreover, TLR3-mediated cell death involves the activation of caspases and engages both extrinsic and intrinsic apoptotic pathways.

Conclusion: TLR3 protein can be expressed in human melanoma cells, where it can deliver proapoptotic and antiproliferative signaling. Altogether, these results suggest that TLR3 agonists represent very promising adjuvants for cancer vaccines not only based on their well-described immunostimulatory properties, but also due to their newly identified cytostatic and cytotoxic effects directly on tumor cells.

The first report demonstrating the involvement of the Drosophila Toll receptor in immunity (1) led to the identification of at least 11 different mammalian Toll-like receptors (TLR). This evolutionarily conserved family of receptors plays a key role in the detection of invading pathogens and in the mounting of the antimicrobial immune response (see ref. 2 for review). Ligand specificity and downstream signaling triggered by ligand recognition have been extensively studied for most of the TLRs. Indeed, TLRs have been shown to recognize pathogen-associated molecular patterns, including lipid-based structures such as mycobacterial lipopeptides (TLR2) or lipopolysaccharide (TLR4), or microbial genetic material such as viral dsRNA (TLR3) or unmethylated CpG islands (TLR9), among others (2).

Binding to their respective ligands leads to a multitude of intracellular downstream events that culminates in cell activation and results in the induction of cytokine secretion and resistance against pathogens (3). However, these signaling cascades can also result in the triggering of cell death (4), which is another way of protecting the host against multiplication and spreading of intracellular microbes (5). Due to the crucial role of TLR in pathogen detection and in the mounting of antimicrobial immune responses, most of the studies done to understand TLR biology have focused on immune cells. These studies have unequivocally established a major immunostimulatory role for TLR ligands, and therefore suggested that these microbial compounds could act as efficient adjuvants in vaccine formulation, most notably in the context of antitumor vaccination (6). Animal tumor models have indeed established the potency of different TLR agonists to enhance the immune responses triggered by vaccines (7), and these results have led to the design of human clinical trials using TLR agonists, such as CpG, as adjuvant for antitumor immunotherapy (8). Similarly, poly(I:C), which is a synthetic analogue of dsRNA and a ligand of TLR3 (9), has been shown to be a potent adjuvant in melanoma (10) and human cervical cancer xenograft (11) murine tumor models. In particular, this TLR3 agonist was shown to directly activate natural killer cells (12), to strongly enhance antigen-specific CD8+ T cells responses (10) and to promote antigen cross-priming by dendritic cell (13).
It is now clear that TLR expression is not restricted to immune cells, but also shared with many different cell types, including endothelial and epithelial cells (14, 15). More importantly, this expression seems to be conserved in the corresponding transformed cells, as mRNA coding for TLR3 and several other TLR have been detected in human and murine tumor cell lines, including B16 melanoma cells (16, 17). Interestingly, although triggering TLR4 in murine colonic cancer cell line MC26 leads to immune evasion mediated by the inhibition of T and natural killer cell activities (16), engagement of TLR3 expressed in human breast tumor cells induces massive apoptosis and proliferation blockade (17). Moreover, the latter in vitro data correlate with the results of a retrospective immunohistologic study on breast cancer patients treated with poly(A:U), a synthetic TLR3 agonist, indicating that only patients with TLR3-positive tumors benefited from the treatment and showed increased overall survival (18).

Scarc functional data regarding the biological outcome of the engagement of tumor-expressed TLR are available. This issue is of major importance because TLR agonists are currently studied in many immunotherapy clinical trials as vaccine adjuvants. In the present work, we show that human melanoma cells can express functional TLR3 protein and that its engagement by TLR3 agonists can lead to caspase activation and tumor cell death when combined to pretreatment with IFNα or cycloheximide (an inhibitor of protein synthesis). These data thereby show that TLR3 agonists represent very promising adjuvants for cancer vaccines not only based on their well-recognized immunostimulatory properties, but also due to their direct proapoptotic effect on tumor cells.

**Materials and Methods**

**Cells and reagents.** Melanoma cell lines were derived at the Ludwig Institute for Cancer Research (Lausanne, Switzerland) from patients undergoing surgical interventions at the Centre Hospitalier Universitaire Vaudois (Lausanne), in accordance with the local bioethics protocols and with informed consent from the patients. Cells were grown in RPMI (Invitrogen Life Technologies) supplemented with 10% FCS (Sigma-Aldrich). Normal human melanocyte cells were obtained from PromoCell and cultivated in melanocyte growth medium (PromoCell) as described by the manufacturer. Poly(I:C) was purchased from InvivoGen and showed no endotoxin contamination (<0.2 ng/mL as determined with the Limulus test). Cycloheximide was from Sigma-Aldrich, and IFNα (Intron A) was from Schering-Plough.

**Apoptosis, proliferation, and cell recovery.** Melanoma cells were plated at 10^4 per well in 96-well plates, and after 6 h adherence, cells were treated with IFNα at the indicated doses for 18 h. After this pretreatment, equal volume of poly(I:C) was added to the wells to get the indicated final concentrations, thereby halving the concentration of IFNα in the wells for the subsequent 96 h of culture (unless otherwise stated). Cell viability was then determined using standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) staining method. Briefly, cells were stained for 2 h at 37°C with 0.5 mg/mL MTT and then lysed in methanol before reading absorbance at 550 nm. For apoptosis measurement, cells were pretreated with either 50 μg/mL cycloheximide for 2 h or 1,000 IU/mL IFNα (unless otherwise stated) for 18 h, before addition of poly(I:C) in an equal volume (final concentration 50 μg/mL, unless otherwise stated). Cycloheximide (25 μg/mL) or IFNα (500 IU/mL) were kept in the culture medium during poly(I:C) treatment. After treatment, apoptosis was assessed by Annexin V staining according to manufacturer’s instruction (BD PharMingen) and subsequent fluorescence-activated cell sorting analysis (FACS Calibur, BD Biosciences). Mitochondrial membrane potential was analyzed by flow cytometry after 20-min incubation at 37°C with 5 mM DiOC6(3), from Molecular Probes. Proliferation was measured with the anti-bromodeoxyuridine (BrdUrd) FITC-conjugated antibody set (BD PharMingen) after 5 h pulse with 10 μg/mL BrdUrd (Sigma-Aldrich), according to the manufacturer’s instructions. All statistics were done using the two-ratio Student’s t-test.

**Lentivirus-mediated RNA interference.** Lentivirus-mediated gene silencing was done as described in detail elsewhere (19). Briefly, oligonucleotides coding for short hairpin RNA (shRNA) targeting human TLR3 (target sequence 5’-CAACATAGCCACATATAAT-3’, corresponding to nucleotides 1,547 to 1,565 of TLR3 coding sequence) were annealed and ligated into pBglII-HindIII restriction sites of the pSuper vector (kind gift of R. Agami, NKI, Amsterdam, the Netherlands). This fragment was then excised together with the H1 promoter from pSuper using BamHI and SalI, and subcloned into the corresponding restriction sites in pBAB286 lentivirus vector (kind gift from R. Iggo, Swiss Institute for Experimental Cancer Research, Eparlices, Switzerland) to generate pBAB286-siTLR3. A similar technical approach was used to generate pBAB286-siLamin, designed to knock down Lamin A/C expression (20). pBAB-siLamin or pBAB286-siTLR3 (20 μg) were then transfected into HEK293 cells, together with the second-generation packaging plasmids (5 μg of pMD2-VSVG and 15 μg of pCMV-R8:91; ref. 21), and lentivirus-containing supernatants were collected 48 h postinfection, filtered, and snap frozen. Melanoma cells were infected with the lentivirus preparation, and the stably infected cells were selected with puromycin (1.25 μg/mL) for 48 h, to get silLamin or siTLR3 Me 260 cells.

**Immunoprecipitations and Western blotting.** Melanoma cells were lysed in the presence of protease inhibitors (Roche) in 1% NP40 containing buffer (150 mmol/L NaCl, 50 mmol/L Tris, 1 mmol/L EDTA, 0.02% NaN3) for 30 min on ice, and nuclei-free lysates collected by centrifugation at 13 krpm for 20 min at 4°C. Cleared lysates (containing 3 mg proteins) were first precleared for 2 h at 4°C in the presence of Protein-G Sepharose beads (Pierce) and rabbit serum, and then incubated for 20 h at 4°C with either preimmune rabbit serum (as a negative control) or 10 μg/mL rabbit anti-TLR3 polyclonal antibody (TLR3-P; ref. 22) in the presence of Protein-G Sepharose. Beads were washed five times in lysis buffer, and immunoprecipitates were eluted by boiling in SDS-PAGE sample buffer containing 2% β-mercaptoethanol, and separated on SDS-PAGE gels before transferring onto nitrocellulose membrane. After 2 h blocking with 5% nonfat milk, membranes were then immunoblotted overnight with guinea pig anti-TLR3 antibodies (TLR3-IC; ref. 22). Proteins were revealed with peroxidase-conjugated goat anti–guinea pig antibody (Milan Analytica AG) and visualized with SuperSignal West Femto system (Pierce). To ensure equal loading, aliquots of supernatants from immunoprecipitation were loaded on SDS-PAGE gels, transferred onto nitrocellulose membranes that were then immunoblotted with anti β-tubulin antibodies (Sigma-Aldrich), further detected with peroxidase-conjugated sheep anti-mouse antibodies (Amersham). Antibodies to Bcl, Caspase-3, caspase-8, and caspase-9 (Cell Signaling); X-IAP (BD Bioscience); FLIP and Noxa (Alexis Biochemicals); A20 (Imgenex); and Bcl-2 and Bax (Upstate) were diluted as indicated by manufacturers.

**Detection of TLR3 expression by flow cytometry.** TLR3 expression was detected by flow cytometry using standard techniques, with biotinylated TLR3.7 antibody and streptavidin phycoerythrin (eBioscience) either at cell surface or intracellularly after fixation and permeabilization with the Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer’s instructions. Stainings were analyzed with FACSCalibur device and CellQuest software.

**Determination of interleukin-6 secretion.** Levels of interleukin-6 in culture supernatants were determined by ELISA using the interleukin-6 Quantikine kit (R&D Systems) according to manufacturer’s instructions.
Results

Some human melanoma cells express TLR3. Several murine tumor cell lines, including the murine melanoma cells B16, have been shown to express mRNA coding for different TLR, including TLR3 (16). We first determined whether TLR3 is expressed in human melanoma cells. Eight melanoma cell lines, including two derived from primary melanoma (Me 300 and T921A), were therefore analyzed by immunoprecipitation followed by Western blotting. Although no protein was immunoprecipitated with the rabbit control serum (data not shown), TLR3 protein was detected as a band of ~120 kDa in five of the eight cell lines (namely Me 300, Me 260, Me 290, NA8-MEL, and T331A; Fig. 1A). Thus, a significant proportion of melanoma cell lines constitutively express TLR3 protein. Moreover, comparable amounts of TLR3 were also detected in cultured normal human melanocytes (Fig. 1B), suggesting that TLR3 expression by human melanoma cell lines is lineage rather than transformation dependent.

Flow cytometry analysis was done on both Me 260 and Me 300 cells to determine TLR3 cellular localization, either at steady state or after 18 h culture in the presence of IFNα. The TLR3 antigen could not be detected at the cell surface. However, it was detectable intracellularly at steady state and slightly unregulated upon exposure to IFNα (Fig. 1C), in good agreement with biochemical data (see below). Of note, the expression levels detected by flow cytometry were both weak and similar between the two cell lines, whereas immunoprecipitation clearly revealed higher amounts of TLR3 protein in Me 300 cells than in the Me 260 cells. This suggests that the latter technique may be more sensitive for this application than flow cytometry.

The TLR3 agonist poly(I:C) can alter the viability of melanoma cells. Having shown that TLR3 is expressed in several melanoma cell lines, we then tested in the responsiveness of the cells to the TLR3 agonist poly(I:C), herein used as a surrogate of viral dsRNA. As a first approach, tumor cell viability was assessed after a 3-day exposure to various concentrations of the ligand. As presented in Fig. 2A, poly(I:C) was able to clearly affect viability in one cell line and marginally in two other cell lines. This effect could be synergistically enhanced by pretreatment with low doses of IFNα. Indeed, in four of six melanoma cell lines analyzed, the combination of IFNα and poly(I:C) reduced cell viability with a magnitude ranging from 40% to 90% compared with the control condition treated only with IFNα, which alone exerted only a very limited effect on cell viability (i.e., <10%; data not shown). Surprisingly, this effect did not correlate with TLR3 expression levels, as the cell line T921A, devoid of detectable TLR3, was shown to be very sensitive to the combination IFNα + poly(I:C), whereas NA8-MEL cells, clearly expressing TLR3 protein, were resistant.

To more precisely characterize the biological mechanisms involved in poly(I:C)-induced toxicity, we analyzed whether the agonist was able to trigger apoptosis in melanoma cell lines. By itself, poly(I:C) did not induce significant apoptosis in the cell lines tested (Fig. 2B). However, when combined to IFNα, it triggered apoptosis in four of the six cell lines, NA8-MEL and Me 290 being mostly resistant to this treatment (Fig. 2B). Moreover, it has been described that, reminiscent of tumor necrosis factor receptor–mediated cytotoxicity, TLR4-induced apoptosis in endothelial cells is inhibited by protective cellular factors that can be suppressed by treatment with protein synthesis inhibitors such as cycloheximide (23). Similarly, when poly(I:C) was added after pretreatment with cycloheximide, we observed a significant cell death in melanoma cells, albeit at different levels depending on the cell line (Fig. 2C).

Thus, it seems that when combined to either type I IFN or cycloheximide, the TLR3 agonist poly(I:C) is able to reduce cell survival and to trigger apoptosis in more than half of the human melanoma cell lines analyzed, although with variation between cell lines.

Characterization of poly(I:C)-induced cell death. The melanoma cell lines Me 260 and Me 300 were further used to study in greater details the proapoptotic effect of TLR3 agonist in melanoma cells. Kinetic studies revealed that the combination of poly(I:C) and low dose of IFNα (10 IU/mL) considerably alters cell survival in a time- and dose-dependent manner, whereas both individual treatments were much less effective (see Fig. 3A). Moreover, the proapoptotic effect of poly(I:C) combined with IFNα (see Fig. 3B) or cycloheximide (see Fig. 3D) was shown to be dose dependent, with doses of TLR3 agonist as low as 500 ng/mL inducing significant apoptosis when combined to cycloheximide. Interestingly, the primary melanoma cell line Me 300 was much more sensitive to IFNα priming, as low doses (10 IU/mL) were enough to render cells sensitive to the poly(I:C) proapoptotic effect, whereas higher doses were required for the metastatic melanoma cell line Me 260. Of note, similar doses of IFNα were required for Me 260 cells to display poly(I:C)-induced interleukin-6 secretion, which mirrored the induction of apoptosis (Fig. 3C).

Fig. 1. TLR3 protein is constitutively expressed in several human melanoma cell lines and in normal melanocytes. A, lysates from the indicated melanoma cell lines were subjected to immunoprecipitation with the anti–TLR3-P antibody. Immunocomplexes were separated under reducing conditions in 9% SDS-PAGE gel and immunoblotted with anti–TLR3-IC antibody. Aliquots (10 µL) of the immunoprecipitation supernatants were separated on 10% SDS-PAGE gel and immunoblotted with anti–β-tubulin antibody to ensure equal quantities of starting material. B, normal human melanocytes (NHM) and Me 260 lysates were analyzed as described above for TLR3 protein expression. C, Me 260 and Me 300 cells were analyzed for TLR3 expression by flow cytometry as described in Materials and Methods using the TLR3.7 antibody. Filled area, isotype control; bold line, TLR3 staining. Results presented are representative of two independent experiments.
Fig. 2. Poly(I:C) synergizes with IFNα and cycloheximide to alter melanoma cells survival. A, human melanoma cells were plated at equal number. After pretreatment with the indicated doses of IFNα for 18 h, cells were further incubated for 3 d without (white columns) or with poly(I:C) (5 μg/mL, gray columns; 50 μg/mL, black columns). Cell viability was then assessed by MTT staining as described in Materials and Methods. Results are expressed as percentage of control [i.e., not treated with poly(I:C)]. Data shown are representative of three independent experiments, and SDs were calculated from triplicates. B, human melanoma cells were left untreated (white columns) or pretreated with IFNα at 1,000 IU/mL for 18 h (black columns) then further incubated with or without poly(I:C) (50 μg/mL) for another 24 h. Apoptosis was measured using Annexin V staining as described in Materials and Methods, and results were expressed as percentage of apoptotic cells in the culture. SDs were calculated from three independent experiments. C, human melanoma cells were left untreated (white columns) or pretreated with cycloheximide at 50 μg/mL for 2 h (black columns), then further incubated for 24 h in the presence or absence of 50 μg/mL poly(I:C). Annexin V staining was done as in (B). SDs were calculated from three independent experiments.
We also thought to investigate whether a modulation of cell proliferation would also account for the reduced cell number observed upon poly(I:C) treatment (Fig. 2A and 3A). BrdUrd incorporation was therefore measured, and showed that poly(I:C) significantly reduced cell proliferation in both Me 260 and Me 300 cell lines, either alone (for Me 300) or when combined to IFNα (Fig. 3E).

Altogether, these results show that poly(I:C) can induce time- and dose-dependent apoptosis and inhibition of proliferation in melanoma cells when combined to either cycloheximide or IFNα.

Poly(I:C)/IFNα treatment induces TLR3 expression and caspase activation and modulates the expression of apoptosis-related molecules. As type I IFNs are known to induce or increase TLR3 protein expression in many cell types (22), we investigated whether this would also be the case for melanoma cells. IFNα was found to increase TLR3 protein expression in both Me 260 and Me 300 cell lines (Fig. 4A). Interestingly, poly(I:C) alone induced a strong up-regulation of its receptor in Me 300 cells but not in Me 260 cells. This might explain why poly(I:C) alone was able to alter the viability of Me 300 cells. In Me 260 cells, the combination IFNα + poly(I:C) resulted in a strong and synergistic induction of TLR3 protein. This induction is likely to account, at least partly, for the priming effect of IFNα to poly(I:C)-induced cell death.

Caspases are major players in the apoptotic processes. When combined to IFNα pretreatment, poly(I:C) induced a strong activation of the extrinsic caspase-8, and, to a lower extent, of the intrinsic caspase-9 (Fig. 4B). Interestingly, the executioner caspase-3 was also activated by poly(I:C) alone in both Me 260 and Me 300 cell lines, although to a much lower extent than when combined to IFNα. Accordingly, poly(ADP-ribose) polymerase cleavage, a hallmark of caspase-mediated apoptosis, was observed in both Me 260 and Me 300 cell lines when poly(I:C) was combined to IFNα (data not shown).

The modulation of expression of proapoptotic and antiapoptotic factors was also studied in melanoma cells after poly(I:C) treatment. Bcl-2 expression was found to be significantly decreased in both cell lines by the combined treatment (Fig. 4C), strongly suggesting that the mitochondrial apoptotic pathway might actually be involved in poly(I:C)-induced cell death. No cleavage of Bid could be observed, and Bax expression was not changed. Interestingly, processing of caspase-3 into the active 20 kDa fragment was observed upon treatment with poly(I:C) alone in both cell lines without concomitant apoptosis (Fig. 4B). Further processing (i.e., activation) into smaller fragments was observed mainly when the cells were pretreated with IFNα, which led to cell death, suggesting the release by IFNα of an apoptosis blockade upstream of caspase-3. X-IAP is an inhibitor of apoptosis that binds to and inhibits activated caspase-3 and caspase-9. Cleavage of X-IAP into its inactive 29 kDa form was shown to occur in Me 260 and Me 300 cells upon IFNα + poly(I:C) treatment, and was readily observed in Me 300 upon treatment with IFNα or poly(I:C) alone.

Antiapoptotic factors such as cFLIP (mainly the short isoform) and the zinc finger protein A20 were induced by poly(I:C) treatment (Fig. 4C). Interestingly, induction was maximal in conditions inducing apoptosis, namely poly(I:C) + IFN in both Me 260 and Me 300, and also upon poly(I:C) treatment alone in Me 300.

Altogether, these results show that the combination of poly(I:C) and IFNα activates caspases and affects apoptosis regulatory molecules, either decreasing (Bcl-2 and X-IAP active form) or increasing (FLIP and A20) their expression.

TLR3 is involved in poly(I:C)-induced melanoma cell death. Several receptors have been described to bind to or at least to mediate the effect of dsRNA in the cell, such as TLR3, PKR, or MDA-5 (9, 24, 25). Because TLR3 protein was detected in melanoma cells, we next asked whether the proapoptotic effect of poly(I:C) in these cells could be mediated by TLR3. We produced a lentivirus designed to stably express a TLR3-specific siRNA to knock down the receptor expression. A lentivirus designed to silence Lamin A/C expression was used as control. Both Me 260 and Me 300 cells were efficiently transduced with shRNA-expressing viruses, as judged by antibiotic resistance. Me 260 cells transduced with the lentivirus expressing TLR3 shRNA (siTLR3 cells) exhibited a dramatic inhibition of TLR3 expression compared with untransduced cells or cells infected with the lentivirus expressing Lamin A/C–specific shRNA (siLamin cells), as shown by immunoprecipitation and Western blotting (see Fig. 5A). Unfortunately, the level of TLR3 expression was not significantly reduced in Me 300 cells. This may be due to the very high level of TLR3 expression in these cells (Fig. 1 and data not shown). We thus focused on the Me 260 cells for the remaining experiments. It has been previously reported that the TRIF signaling pathway may be of biological relevance for tumor growth (17, 26). The steady-state proliferation of siTLR3 melanoma cells was therefore compared with that of wild-type and siLamin cells by means of BrdUrd incorporation analysis. As shown in Fig. 5B, inhibition of TLR3 expression by small interfering RNA (siRNA) did not alter by itself the rate of cellular proliferation, which proved to be equivalent in parental, siLamin and siTLR3 Me 260 melanoma cells. The effect of poly(I:C) on cell viability (Fig. 2A) was then analyzed in control and TLR3 knockdown cells (Fig. 5). Similar to nontransduced cells, a synergy between IFNα and poly(I:C) was apparent in cells, siLamin, used as control. In contrast, siTLR3 cells were totally resistant to poly(I:C) toxicity, clearly indicating the involvement of TLR3 in this phenomenon (Fig. 5C). Apoptosis induced by the combination of poly(I:C) with either cycloheximide or IFN-α was also significantly reduced in TLR3 knockdown Me 260 cells compared with unmanipulated (control) or siLamin cells (Fig. 5D). Similarly, the dose-dependent inhibition of cellular proliferation observed upon poly(I:C) + IFNα treatment was abolished in TLR3 silenced Me 260 (Fig. 5E), clearly demonstrating that the antiproliferative effect of poly(I:C) was also mediated by TLR3. Last, because we showed that the apoptotic mitochondrial pathway was triggered by the combination of poly(I:C) + IFNα (Fig. 4B), we sought to investigate whether this would also be dependent on TLR3. The mitochondrial transmembrane potential ($\Delta \Psi$) decreased when untransduced or Lamin knockdown Me 260 cells were treated with poly(I:C) + IFNα, whereas it remained high in treated TLR3 knockdown cells (Fig. 5E). These results indicate that the combination of poly(I:C) and IFN-α not only triggered mitochondrial depolarization, thereby confirming the involvement of the intrinsic apoptosis pathway, but also establish that the depolarization event is TLR3 dependent. Comparable results were obtained using cells from two independent infections. Altogether, these results established a key role for TLR3 in poly(I:C)-triggered apoptosis of human melanoma cells.
Fig. 3. Poly(I:C)-induced toxicity is time and dose dependent. A, Me 260 and Me 300 melanoma cells were preincubated with 10 IU/mL IFNα for 18 h and then further cultured in the absence or presence of poly(I:C) (5 or 50 µg/mL) for up to 5 d. Cell number was determined by MTT assay after IFN pretreatment (set as day 0) and at days 3, 4, and 5 of poly(I:C) treatment. Values measured at day 0 were arbitrarily set at 1. Results are expressed as arbitrary units compared with cells not treated with poly(I:C). SDs were calculated from triplicates, and data shown are representative of two independent experiments. B, Me 260 and Me 300 melanoma cells were preincubated with the indicated dose of IFNα for 18 h. Poly(I:C) was then added at different concentrations and apoptosis measured 24 h later by Annexin V staining as described in Materials and Methods. Columns, percentage of apoptotic cells in the culture; bars, SD. Calculated from three independent experiments. C, Me 260 cells were treated as in (A), and interleukin-6 (IL-6) levels were determined by ELISA in culture supernatants after 3 d of poly(I:C) treatment. SDs were calculated from triplicates. D, Me 260 and Me 300 melanoma cells were pretreated with cycloheximide at 50 µg/mL for 2 h. Cells were further incubated for 24 h in the absence (white columns) or presence of poly(I:C) at the indicated doses (black columns). Annexin V staining was done as described in Materials and Methods. SDs were calculated from three independent experiments. E, Me 260 and Me 300 melanoma cells were pretreated with the indicated dose of IFNα for 18 h then further incubated in the absence (white columns) or presence of poly(I:C) (50 µg/mL, black columns) for 24 h. Cell proliferation was then analyzed by BrdUrd incorporation as described in Materials and Methods. Results are expressed as percentage of proliferating cells in a culture compared with untreated cells (considered as 100% of proliferation). SDs were calculated from three independent experiments.
Discussion

The results presented here show that a large proportion of human melanoma cell lines express TLR3 at the protein level. Moreover, we show that the TLR3 agonist poly(I:C), when added after pretreatment with either cycloheximide or type I IFN, is able to induce significant \textit{in vitro} cell death and proliferation blockade through TLR3 signaling in these melanoma tumor cells. These data confirm and extend the recent observations on the proapoptotic properties of poly(I:C) upon TLR3 engagement in a human breast cancer cell line (17). Moreover, data pointing to a direct proapoptotic function of TLR3 have also been reported by other groups. Transfection-based studies have identified TRIF as a molecular adaptor endowed with proapoptotic properties (27), and TRIF-deficient macrophages were shown to be resistant to cell death induced by both lipopolysaccharide and dsRNA (28). There is also evidence that TLR3 may be involved in cell death of pancreatic \( \beta \) cells in the course of type 1 diabetes mellitus (29, 30). The present results add to the growing body of evidence that multiple TLR can induce cell death, as shown for TLR2, TLR4, and TLR5 (4, 23, 31). With regard to the TLR3-dependent effect on proliferation, a clear cytostatic effect of poly(I:C) on human melanoma cell lines has already been described (32). Although TLR3 involvement was not addressed, it is likely that results from that study are closely related to those described here.

The subcellular localization of TLR3 is still a matter of debate. It has been suggested to be expressed at the cell surface in fibroblasts (33), but recent biochemical evidence indicate that TLR3 requires an acidic environment to mediate poly(I:C)-induced signaling (34), which is consistent with the hypothesis that the recognition of poly(I:C) by TLR3 takes place in intracellular vesicles, very likely of endosomal origin (35). Our own results also suggest an intracellular localization for TLR3 in melanoma cells (Fig. 1C). Along these lines, the existence of a still unidentified, surface-expressed receptor that would transfer TLR3 agonists from the extracellular space to intracellular vesicles where binding to TLR3 would occur has been proposed (36). Interestingly, recent data described CD14 as a potential carrier receptor that would shuttle dsRNA from cell surface to intracellular TLR3-containing, endolysosomal-like structures (37). dsRNA entry into the cell has also been recently proposed to be mediated by a yet unidentified pattern recognition receptor linked to the endocytic pathway and to the RNA silencing machinery (38).

Whether the differential expression of CD14 or other unidentified dsRNA receptors is implicated in the variability in apoptosis induced in tumor cells by dsRNA remains an interesting issue to address, that could potentially explain why NA8-MEL and Me 290 melanoma cell lines were not induced to undergo apoptosis when exposed to type I IFN and poly(I:C) despite the fact that they up-regulated TLR3 protein expression upon IFN\( \alpha \) treatment (data not shown). In this regard, we observed that Me 290 cells were resistant to cisplatin-induced apoptosis, thereby suggesting that at least in this cell line the resistance to type I IFN and poly(I:C) was due to a general defect in apoptotic pathways rather than to a specific issue regarding dsRNA-induced cell death. Whether other molecules involved (or not) in TLR3 signaling are lacking in cells resistant to the IFN\( \alpha \) + poly(I:C) combination remains to be determined. Along this line, Fisher and colleagues (39, 40) showed that the IFN-inducible RNA helicase MDA-5, which is a key player in poly(I:C)-induced responses (25), is also able to trigger both apoptosis and inhibition of proliferation when ectopically expressed in melanoma cells. Whether TLR3 and MDA-5 have differential roles or cooperate for the induction of such proapoptotic responses in tumor cells obviously deserves further investigations.

In the present study, type I IFN was not toxic by itself for melanoma cells, but instead was required as priming agent.

![Fig. 4. Effect of poly(I:C) and IFN\( \alpha \) on TLR3 and apoptosis-related molecules in melanoma cells. Melanoma cells were preincubated with or without IFN\( \alpha \) \( (10^5 \text{ IU/mL}) \) for 18 h, and then further cultured for 24 h in the presence or absence of poly(I:C) \( (50 \mu\text{g/mL}) \). Cell lysates were prepared and either immunoprecipitated (A) as in Fig. 1, or directly analyzed by Western blotting (B and C) using antibodies to the indicated proteins. \( \beta \)-Tubulin is shown as loading control. Casp, cleaved caspase; n.s., nonspecific band.](www.aacrjournals.org)
for dsRNA-triggered cell death, reminiscent of its role in TLR3-induced apoptosis in breast cancer cells (17) and in pancreatic β cell death (30). The molecular mechanisms underlying this proapoptotic synergy between type I IFN and dsRNA are unclear, although this phenomenon has already been observed more than 30 years ago (41). Several explanations have been proposed, including up-regulation of proteins involved either in dsRNA signaling (such as PKR, IRF-3, or TLR3; ref. 14) or in apoptosis (such as tumor necrosis factor–related apoptosis-inducing ligand or p53; ref. 42), and modulation of the mitochondrial apoptotic pathway (43) and of the death-inducing signaling complex activity (44). Our results show that IFNα indeed up-regulates TLR3 protein expression in Me 260 and Me 300 melanoma cells (Fig. 2B). The T921A cell line, devoid of detectable TLR3 expression at steady state (Fig. 1A), was also shown to up-regulate TLR3 upon IFNα treatment, thereby explaining its sensitivity to TLR3-dependent IFNα + dsRNA–induced apoptosis (data not shown). Although TLR3 is required for poly(I:C)-induced cell death in melanoma cells, its up-regulation is, however, not sufficient to induce apoptosis. Indeed, similar to sensitive cells, TLR3 up-regulation was observed in Me 290 and NA8-MEL cells (data not shown), both resistant to the combination IFNα + poly(I:C). In addition, X-IAP cleavage and Bcl-2 down-regulation were triggered by IFNα + poly(I:C) treatment in both Me 260 and Me 300 cell lines, and IFNα allowed TLR3-dependent mitochondrial depolarization upon poly(I:C) treatment. One could therefore conclude that IFNα sensitizes melanoma cells to TLR3-induced apoptosis at least in part by facilitating the triggering of the intrinsic apoptotic pathway. This could happen in parallel to engagement of the death receptor pathway that is already weakly activated by poly(I:C) alone in Me 300 cells, but for which melanoma cells elaborated protection strategies such as the induction of A20 or FLIP. It is interesting to note that type I IFN have also been described to sensitize melanoma cells to death receptor–induced apoptosis (45), and this was accompanied by X-IAP cleavage, in a very similar way to what we describe here.

It is also worth noting that most proapoptotic pathways described to be triggered by dsRNA (whether delivered extracellularly or transfected into the cells; refs. 17, 46) or by other TLR agonists (4, 27, 28, 47) involve caspase-8 activation, probably mediated by FADD recruitment to the receptors, into a complex called “dsRNA-death-inducing signaling complex” in the case of dsRNA (46). The kinase Rip1 is also involved in TRIF-induced proapoptotic signaling (27). This all strongly reminds of the well-characterized death receptor signaling, shared by the tumor necrosis factor receptor and CD95/Fas, and would therefore suggest that TLR can indeed be considered as death receptors, at least with respect to their proapoptotic signaling. The parallel could even been drawn further, as both the tumor necrosis factor receptor and the death-inducing TLR seem to induce a prosurvival signaling that counteracts their proapoptotic properties. Indeed, the level of apoptosis induced through the sole triggering of TLR3 is not conspicuous, indicating that yet unidentified cellular factor(s) regulate and attenuate the proapoptotic properties of TLR3. This was shown by the ability of cycloheximide pretreatment to strongly sensitize melanoma cells to dsRNA-induced apoptosis. This situation is reminiscent of the cell death induced by TLR2, TLR4, TLR5 (4, 23, 47), or the tumor necrosis factor receptor (48), where prosurvival factors have to be inhibited to get full-blown activation of the prosapoptotic cascade downstream of the receptors. FLIP is one of such factors, shown to protect endothelial cells against TLR4-induced apoptosis (23) and to be strongly up-regulated in melanoma (49). However, our results show that melanoma cells undergo IFNα + poly(I:C)–induced apoptosis despite cFLIP induction, thereby making it unlikely that FLIP is a determinant prosurvival factor downstream of TLR3. Similarly, the antiapoptotic factor A20, although strongly induced by IFNα + poly(I:C) in Me 300 cells, does not efficiently protect the cells against cell death. Another candidate factor mediating prosurvival signaling is NF-κB. Interestingly, this transcription factor is up-regulated in melanoma and is thought to mediate (at least in part) tumor resistance to cell death (50). Accordingly, we found that the proteasome inhibitor MG-132, which indirectly blocks NF-κB activation by blunting the proteosomal degradation of the inhibitor IκB, strongly sensitized melanoma cells to TLR3-induced apoptosis (data not shown). The identification of the prosurvival molecules that counteract TLR3-induced cell death would be of outstanding interest for clinical applications of TLR3 agonists as adjuvants, because the expression of these factors might indicate whether the tumor would be sensitive (or not) to TLR3-triggered cell death, and could offer opportunities to sensitize tumors that would otherwise be resistant to TLR3 agonists-induced apoptosis.

Melanoma is an aggressive type of cancer that is highly resistant to conventional treatments. The standard adjuvant therapy, consisting of systemic treatment with IFNα (51), is limited by serious side effects. TLR3 agonists have been used in the past to treat cancer patients, showing a good safety and tolerability profile (52). Because TLR3 agonists are well known for their ability to induce strong production of type I IFN (9, 53, 54), one could consider that triggering in situ production of type I IFN by systemic administration of TLR3 agonists could

![Fig. 5.](https://www.aacrjournals.org) Involvement of TLR3 in the proapoptotic effect of poly(I:C) on melanoma cells. A, lentivirus-based system was used to express siRNA duplexes in the melanoma cell line Me 260 as described in Materials and Methods. Lysates from untreated parental cells (control) and cells infected with virus coding for Lamin A/C (siLamin) or TLR3 (siTLR3) specific siRNA were analyzed by immunoprecipitation with TLR3-P antibody and Western blotting with anti–TLR3-IC antibody. β-Tubulin expression was determined in aliquots of immunoprecipitation supernatants to ensure equal loading. B, parental Me 260 cells (white columns) and cells transduced with Lamin (gray columns) or TLR3 (black columns) specific siRNA were plated at equal number, and BrdUrd labeling was done as described in Materials and Methods. Columns, percentage of BrdUrd incorporating cells in the culture; bars, SD, calculated from three independent experiments. C, parental Me 260 cells (control) and cells transduced with Lamin A/C (siLamin) or TLR3 (siTLR3) specific siRNA were plated at equal number and treated with IFNα and poly(I:C) as in Fig. 2A. Cell number was assessed by MTT staining. White columns, no poly(I:C), gray and black columns, 5 and 50 μg/mL poly(I:C), respectively; bars, SD, calculated from triplicates. Results presented are representative of three independent experiments. D, parental Me 260 cells (white columns) and cells transduced with Lamin (gray columns) or TLR3 (black columns) specific siRNA were treated with cycloheximide and poly(I:C) (left) or IFNα and poly(I:C) (right) as described in Fig. 2C and B. Apoptosis analysis was then done using Annexin V staining. SDs were calculated from three independent experiments. E, parental Me 260 cells (white columns) and cells transduced with Lamin (gray columns) or TLR3 (black columns) specific siRNA were treated as in (C). BrdUrd labeling (left) was done as described in Materials and Methods. Results are expressed as percentage of BrdUrd-positive cells compared with untreated cells (considered as 100% proliferation). The mitochondrial membrane potential (Δψm, right) was measured with DiOC6(3) dye as described in Materials and Methods. Results are expressed as percentage of cells with low Δψm. SDs were calculated from three independent experiments.
considerably lower its adverse side effects and contribute to a better targeted therapy by inducing the tumor cells to endogenously produce type I IFN (17, 32). Altogether, considering that TLR3 agonists have been shown to represent very promising adjuvants for cancer vaccines, with respect to their ability to promote antigen-specific CD8 T cells responses (10, 13), our results might open new clinical perspectives for the use of TLR3 agonists as multifunctional adjuvants, able to both directly kill the cancerous cells and increase the innate and specific immune antitumor responses.

References


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