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Direct effect of dsRNA mimetics on cancer cells induces endogenous IFN-β production capable of improving dendritic cell function

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Viral double-stranded RNA (dsRNA) mimetics have been explored in cancer immunotherapy to promote antitumoral immune response. Polyinosine–polycytidylic acid (poly I:C) and polyadenylic–polyuridylic acid (poly A:U) are synthetic analogs of viral dsRNA and strong inducers of type I interferon (IFN). We describe here a novel effect of dsRNA analogs on cancer cells: besides their potential to induce cancer cell apoptosis through an IFN-β autocrine loop, dsRNA-elicited IFN-β production improves dendritic cell (DC) functionality. Human A549 lung and DU145 prostate carcinoma cells significantly responded to poly I:C stimulation, producing IFN-β at levels that were capable of activating STAT1 and enhancing CXCL10, CD40, and CD86 expression on human monocyte-derived DCs. IFN-β produced by poly I:C-activated human cancer cells increased the capacity of monocyte-derived DCs to stimulate IFN-γ production in an allogeneic stimulatory culture in vitro. When melanoma murine B16 cells were stimulated in vitro with poly A:U and then inoculated into TLR3$^{-/-}$ mice, smaller tumors were elicited. This tumor growth inhibition was abrogated in IFNAR1$^{-/-}$ mice. Thus, dsRNA compounds are effective adjuvants not only because they activate DCs and promote strong adaptive immunity, but also because they can directly act on cancer cells to induce endogenous IFN-β production and contribute to the antitumoral response.

Keywords: Dendritic cells · dsRNA · IFN-β · TLR3 · Tumor immunity

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Introduction

Polyinosine–polycytidylic acid (poly I:C) and polyadenylic–polyuridylic acid (poly A:U) are synthetic analogs of viral double-stranded RNA (dsRNA), recognized by both Toll-like receptor 3 (TLR3) and RIG (retinoic acid-inducible gene)-I-like receptors (RLRs) or only TLR3, respectively [1–3]. These receptors are expressed mainly on APCs. Both compounds strongly enhance antigen-specific CD8$^+$ T-cell responses, promoting antigen cross-presentation by dendritic cells (DCs), and directly acting on

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effector CD8$^+$ T cells and natural killer cells to augment IFN-γ release [4–7]. A direct effect of synthetic dsRNA on cancer cells has also been demonstrated, since they are capable of inducing the production of type I IFNs, which in turn promotes the apoptosis of cancer cells through an autocrine signaling loop [8–11].

Both poly I:C and poly A:U are strong inducers of type I IFNs. Type I IFNs can be produced by almost any cell type in the body in response to stimulation of TLR3, RLRs, and many other receptors [12]. Exogenously administered type I IFNs were used with some success (and a substantial number of toxic side effects) in anticancer therapy [13]. In contrast, the role of endogenous type I IFNs in cancer therapy has only recently begun to be elucidated [14–17].

We have recently shown that when murine tumorigenic cancer cells are stimulated in vitro with a TLR4 ligand such as lipopolysaccharide (LPS) prior to their inoculation into TLR4-deficient mice, they yield smaller tumors than those elicited by nonstimulated cells. The apoptosis/proliferation balance of LPS-stimulated cancer cells was neither modified, nor was this effect observed in athymic nude mice [18]. Interestingly, the inhibition of tumor growth observed was associated to the presence of DCs with a more mature phenotype as well as increased frequencies of CD11c$^+$ IL-12$^+$ and CD3$^+$ IFN-γ$^+$ tumor infiltrating cells. Moreover, IFN-β secreted by TLR4-activated tumor cells was involved in improving DC maturation and IL-12 production in vitro. Mechanistic investigations revealed that IFN-β was the critical factor produced by TLR4-activated tumor cells, since tumor growth inhibition was abrogated in IFNAR1-deficient mice lacking a functional type I IFN receptor for binding IFNs [19].

These findings prompted us to investigate if other TLR ligands, known to be stronger inducers of type I IFNs, could also stimulate tumor cells to produce IFN-β and positively contribute to the antitumoral immune response. We focused specifically on TLR3 ligands, currently proposed as effective adjuvants in different therapeutic settings [20, 21]. In the present work, we show that dsRNA-activated murine B16 melanoma cells also produce high levels of IFN-β. Moreover, B16 cells activated in vitro with poly A:U and then inoculated into TLR3-deficient mice elicited smaller tumors. Again, this tumor growth inhibition was abrogated in IFNAR1-deficient mice. Furthermore, poly I:C-stimulated human cancer cell lines can also be a source of IFN-β, at levels that are capable of improving the maturation state of human monocyte derived DCs (MoDCs) and reversing the suppressive effect of tumor cell derived factors on MoDC maturation [22, 23].

Our results demonstrate that dsRNA oligonucleotides are effective adjuvants not only because they activate DCs and promote strong adaptive immunity, but also because they can act directly on cancer cells to induce endogenous IFN-β production. We describe a novel effect of dsRNA synthetics on cancer cells: besides their potential to induce cancer cell apoptosis through the IFN-β autocrine loop, dsRNA-elicited IFN-β production participates in improving DC functionality, which could in turn improve the antitumoral immune response.

Results

Activation of human cancer cell lines by dsRNA analogs induces a type I IFN response

According to our previous results, IFN-β produced by TLR4-activated murine tumor cells improve the maturation and IL-12 production of bone marrow derived DCs (BMDCs), normally impaired in tumor settings [18, 19, 22, 23]. To analyze if other TLR ligands, currently used in clinical settings, could reproduce these findings in a human system, A549 cells were stimulated with poly I:C and poly A:U and then the type I IFN response was analyzed. A549 cells express constitutively *TLR3*, *RIG-1*, and *MDA5* mRNA, which have been shown to be receptors for poly I:C. Upon 24 h of stimulation of A549 cells with poly I:C, an upregulation of the different receptor transcripts was detected. Indeed, *TLR3, MDA5*, and *RIG-1*mRNA expression levels showed a strong upregulation (×20-, ×75-, ×62-fold induction, respectively) (Fig. 1A). Interestingly, an important increase in the transcription of genes from the IFN pathway was observed (Fig. 1A), whereas *IFNa* mRNA was no detected (data not shown). A barely augmented transcription of proinflammatory cytokine genes such as *TNF* and *IL1b* could also be determined (Fig. 1A). As expected, induction of interferon regulatory factor (*IRF*) related genes was paralleled by robust phosphorylation of IRF3 4 h after stimulation with poly I:C (Fig. 1B). Biologically active type I IFNs were measured in culture supernatant after stimulating A549 cells with poly I:C for 24 h (PIC-A549 conditioned medium (CM)). Poly I:C-stimulated A549 cells showed a significative increase compared to nonstimulated cells (400 pg/mL). These results were reproduced (although at lesser extent) when the human prostate adenocarcinoma DU145 cells were similarly stimulated. Indeed, type I IFN increased approximately threefold over nonstimulated DU145 cells (13 pg/mL, Fig. 1C).

Once produced, IFN-β activates its receptors (IFNAR1/2) and recruits JAKs to result in phosphorylation of STAT1 and STAT2. Subsequently, phosphorylated STATs form homo- and heterodimers that are transported into the nucleus, where they serve as active transcription factors [12, 24]. The type I IFN autocrine loop already described was also evident in our experimental setting, since STAT1 phosphorylation was evidenced 24 h after the initial activation of the cells (Fig. 1B).

Altogether, our results indicate that A549 lung and DU-145 prostate adenocarcinoma cells significantly respond to poly I:C stimulation, resulting in a massive upregulation of the levels of IRF-related genes and mainly IFN-β.

Poly I:C-activated tumor cell supernatants activates STAT1 in MoDCs

Next, we examined if IFN-β detected in poly I:C-activated A549 and DU145 cell CM (PIC-A549 and PIC-DU, respectively) could act in a paracrine fashion on DCs. Immature MoDCs were incubated

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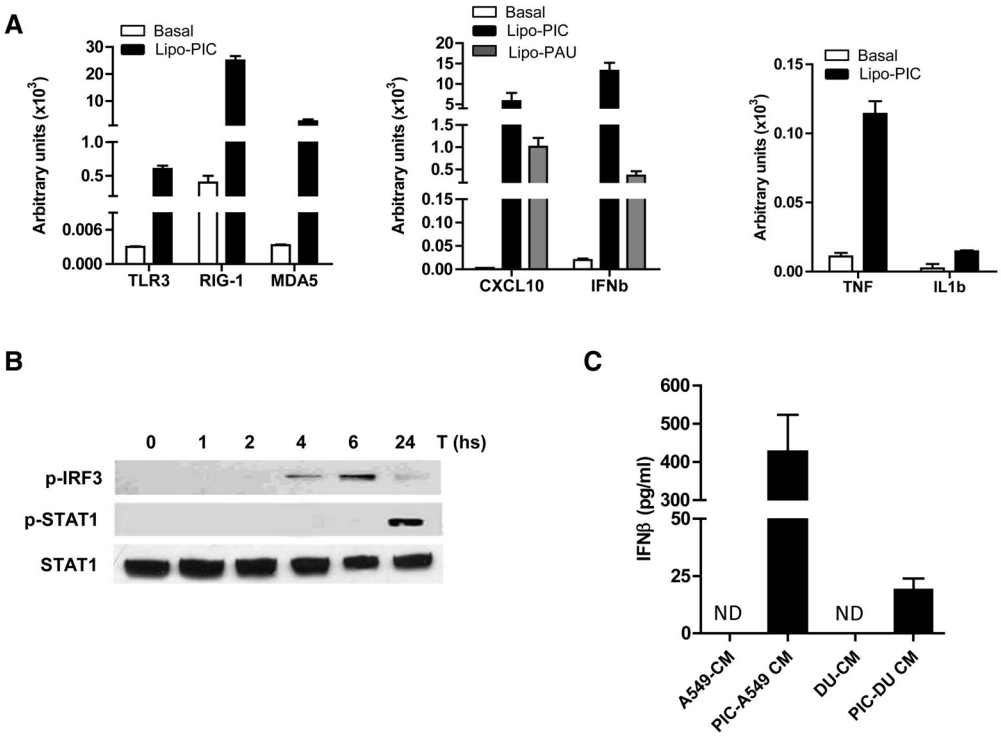

Figure 1. Activation of human cancer cell lines by dsRNA analogs induces a type I IFN response. (A) A549 cells were either mock-transfected (basal) or were stimulated with 0.1 μg/mL of poly I:C complexed to cationic liposome (Lipo-PIC). After 24 h of culture, cells were harvested and *TLR3*, *RIG-1*, *MDA5*, *CXCL10*, *IFNb1*, *TNF*, and *IL1b* mRNA expression was assessed by qRT-PCR analysis. (B) A549 cells were stimulated with Lipo-PIC (0.1 μg/mL) for the indicated time periods. Thereafter, p-IRF3, p-STAT1, and STAT1 protein expression was determined by immunoblot analysis. Data shown are representative of two experiments performed. (C) Presence of type I IFN in conditioned medium (CM) from nonstimulated A549 and DU145 cells (A549-CM and DU-CM, respectively) or from Lipo-PIC-stimulated A549 and DU145 cells (PIC-A549 and PIC-DU CM, respectively). Type I IFN levels were evaluated with the HEK-Blue IFN-α/β cell system and a recombinant IFN-β as standard, 24 h poststimulation with Lipo-PIC. (A, C) Data are shown as mean + SEM of three replicates and are from one experiment representative of three experiments performed.

in PIC-A549 CM or PIC-DU CM for different times and STAT1 phosphorylation was analyzed by Western blotting (Fig. 2A). To rule out the possibility of activation of STAT1 by residual amounts of poly I:C present in PIC-A549 CM, all CMs were treated, before addition to MoDCs, with soluble human recombinant TLR3 that neutralizes the poly I:C activity [25]. As control, MoDCs were stimulated with only poly I:C. Interestingly, STAT1 phosphorylation was detected neither in MoDC incubated with fresh media nor in nonstimulated A549 or DU145 supernatants (A549 and DU-CM, respectively) (Fig. 2A). In contrast, MoDC incubated with PIC-A549 and PIC-DU CM showed strong STAT1 phosphorylation as early as 15 min post addition of the CM. Stimulation of MoDCs with poly I:C alone did not induce STAT1 phosphorylation at the time periods assayed. Similarly, only murine BMDCs cultured with PAU-B16 CM showed STAT1 phosphorylation after 30 min of incubation (Supporting Information Fig. 1A). Given that IFN-β-induced STAT1 phosphorylation is responsible for the CXCL10 production by DCs [12], we also evaluated whether PIC-A549 CM was capable of inducing *CXCL10* mRNA expression in MoDCs. As expected, a strong induction of *CXCL10* mRNA expression was detected only in Mo-CD incubated with PIC-A549 CM (Fig. 2B). These results suggest that MoDCs can be targets of IFN-β present in PIC-A549 or PIC-DU145 CM.

IFN-β produced by tumor cells acts as a positive modulator of DC maturation

Tumor-derived factors significantly inhibit the generation as well as the maturation of DCs [22, 23]. Since type I IFNs and pro-inflammatory cytokines are positive modulators of both phenomena, we hypothesized that IFN-β present in PIC-A549 CM or PIC-DU CM could act as a positive modulator of DC maturation and participate in reversing this inhibited state. To address this hypothesis, MoDCs were first incubated with A549-CM or PIC-A549 CM for 48 h and classical activation markers of DC maturation (CD86 and CD40) were evaluated (Fig. 3). The same experiment was performed using DU-CM and PIC-DU CM. The results obtained using both cell lines were similar: interestingly, PIC-A549 CM and PIC-DU CM are capable per se of significantly enhancing the expression of CD86 and CD40 markers (Fig. 3A and B). When MoDCs were matured with LPS in the presence of A549-CM or DU-CM, the increment of CD86 expression showed a significant drop compared to the increment observed when MoDCs were matured with LPS alone. This inhibitory effect of A549-CM or DU-CM on MoDC maturation was abolished when the CM was originated from PIC-A549 CM or PIC-DU CM. Similar results were observed when a different maturing stimulus, such as the TLR7/8 ligand,

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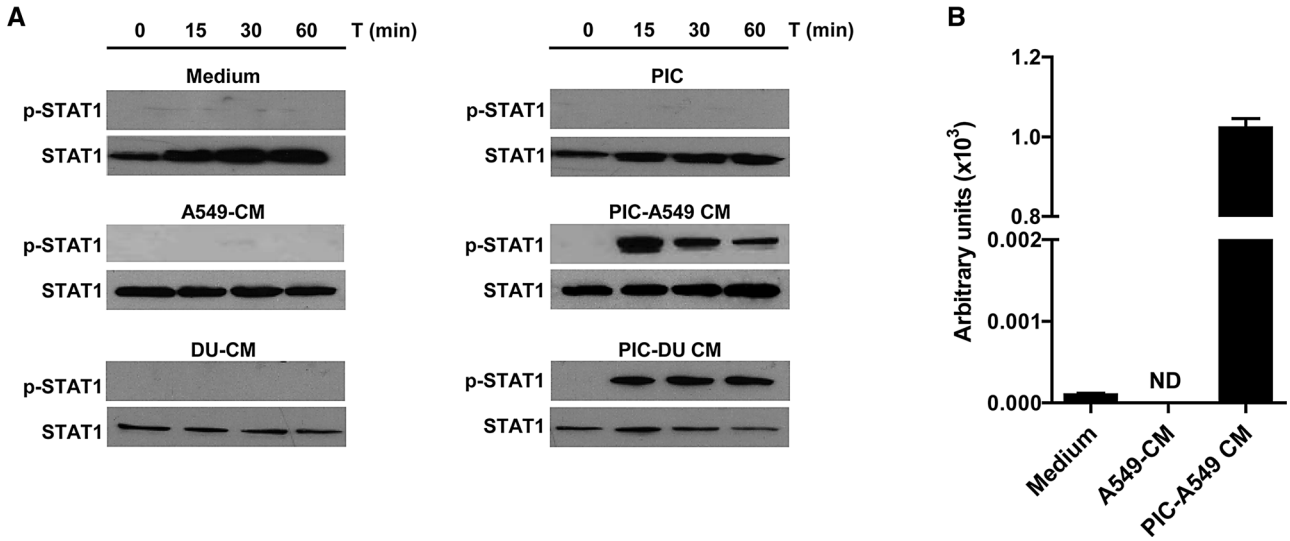

Figure 2. Poly I:C-activated tumor cell conditioned media (CMs) activates STAT1 phosphorylation in MoDCs. (A) Immature MoDCs were incubated with culture medium, poly I:C (PIC; 0.1 μg/mL), A549-CM, DU-CM, PIC-A549 CM, and PIC-DU CM for 0, 15, 30, and 60 min. Cells were then collected and whole-cell proteins were extracted and tested by Western blotting with anti-p-STAT1 and anti-total STAT1 antibodies. Data shown are from one experiment representative of two experiments performed. (B) Immature MoDCs were incubated with culture medium, A549-CM or PIC-A549 CM for 24 h. After 24 h of culture, cells were harvested and *CXCL10* mRNA expression was assessed by qRT-PCR analysis. Data are shown as mean + SEM of three samples and are from one experiment representative of three experiments performed.

R848, was used (Supporting Information Fig. 2A and B). These findings indicate that CM from PIC-stimulated tumor cells is capable by itself of increasing CD86 and CD40 expression on MoDCs and also of subverting the inhibitory effect of A549-CM and DU-CM on LPS-induced maturation.

To check if IFN-β present on PIC-tumor CM was responsible for the effect observed, a neutralizing anti-IFN-β was added to the different CM 1 h before incubating them with MoDCs. As shown in Figure 3C, neutralizing IFN-β completely abrogated the increment in the expression levels of CD40 and CD86 observed when MoDCs were incubated with PIC-A549 CM and PIC-A549 CM + LPS.

Next, we analyzed the ability of A549-CM and PIC-A549 CM to modulate IL-12 secretion. It is generally accepted that DCs need to be stimulated simultaneously with a combination of TLR ligands in the presence of endogenous levels of type I IFN in order to produce biologically active levels of IL-12p70 [26]. In accordance with this idea, neither poly I:C nor LPS stimulation of MoDCs induced high levels of IL-12. Whereas PIC-A549 and PIC-DU CMs were capable per se of increasing CD86 and CD40 levels, they did not induce IL-12 production by MoDCs. In contrast, when MoDCs were stimulated with LPS or R848 in the presence of PIC-CM, a strong increase in IL-12 levels was measured (Fig. 4A and B and Supporting Information Fig. 2C), indicating that IFN-β present in the CM could be acting synergistically with a TLR ligand to induce this crucial cytokine.

We then tested the capacity of MoDC matured in the presence of PIC-A549 CM to stimulate allogeneic PBMCs to produce IFN-γ secretion (Fig. 3C and D). MoDCs were matured with a TLR ligand (LPS or R848) in the presence of A549-CM or PIC-A549 CM. As expected, when MoDCs were matured by only one TLR ligand, either LPS or R848, they were capable of inducing the production of IFN-γ in allogeneic culture supernatants (~1000 and 4000 pg/mL, respectively) (Fig. 4C and D). Interestingly, when MoDCs were exposed to the TLR ligand in the presence of A549-CM (or DU-CM, data not shown), levels of IFN-γ produced in the allogeneic cultures significantly drop. Interestingly, IFN-γ levels are restored or are even higher when the PBMCs were cocultured with MoDCs that were matured in the presence of PIC-A549 CM simultaneously with a TLR ligand (Fig. 4C and D). Similar results were obtained when we evaluated the proliferation of allogeneic PBMC cocultured with MoDC activated under the different experimental conditions (Supporting Information Fig. 3).

This increase in IFN-γ production is abrogated when a neutralizing anti-IFN-β was added to the culture (Fig. 4E). These results indicate that dsRNA analogs can act on human cancer cells and induce the production of type I IFNs, which in turn can promote an improvement in DC function.

IFN-β produced by dsRNA-activated B16 melanoma cells is involved in tumor growth inhibition

To see if IFN-β produced by dsRNA-activated cancer cells could influence tumor growth, we stimulated murine melanoma B16 cells with poly A:U complexed to polyethylenimine (PEI) for 24 h (PAU-B16). We chose poly A:U because it has been previously reported that it only signals through TLR3 [27]. As reported, B16 melanoma cells express constitutively TLR3 mRNA [20], which was upregulated after 24 h poststimulation with poly A:U (PAU-B16 ×2,5; lipofectamine (Lipo)-PAU ×4,7). In addition, an important increase of *IFNb* gene expression was observed (PAU-B16 ×5; Lipo-PAU ×57) (Supporting Information Fig. 1B and C). IFN-β

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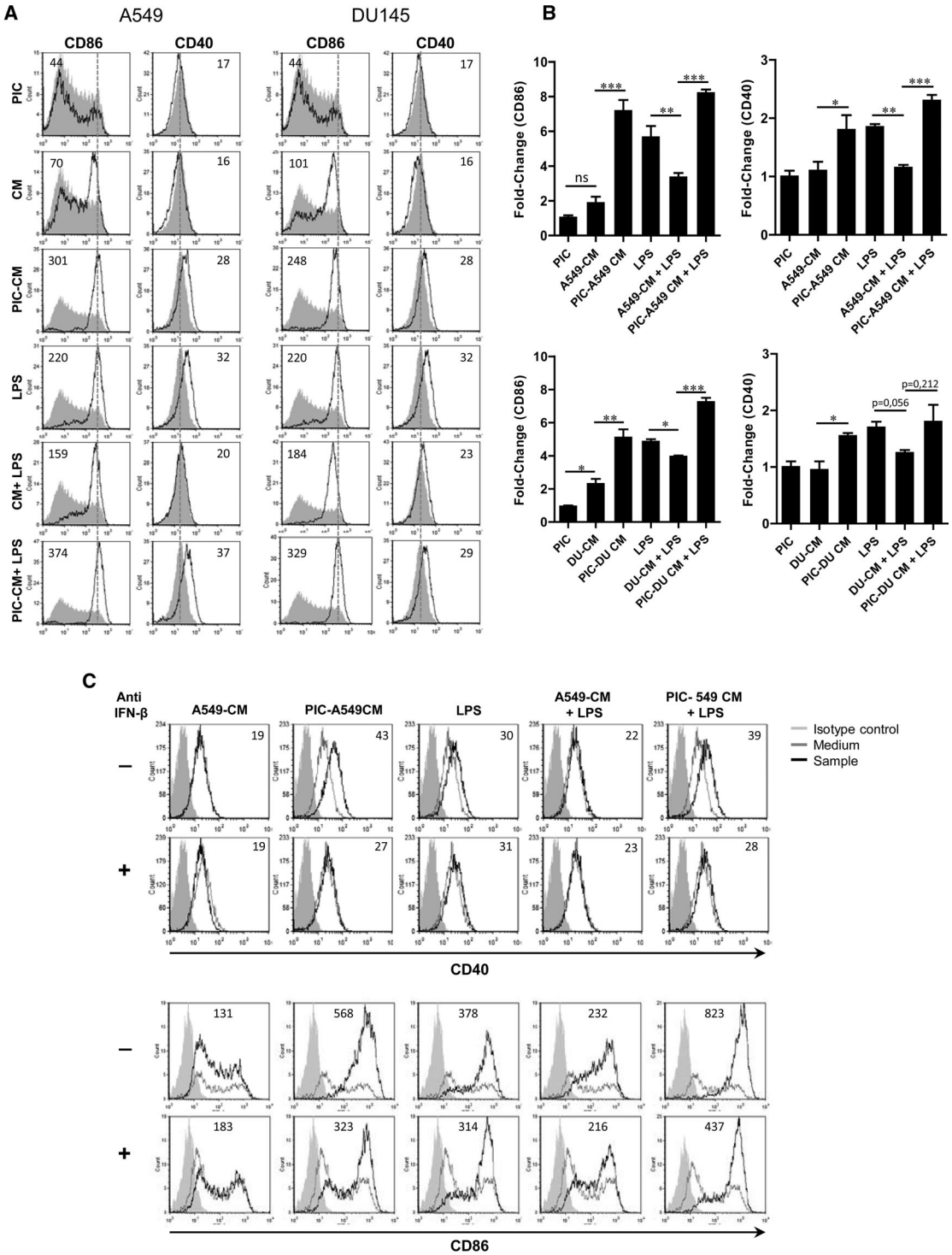

Figure 3. IFN-β produced by poly I:C-activated tumor cells can mature DCs and reverse the suppressive effect of cancer cell derived factors on LPS-mediated MoDC maturation. (A) MoDCs were incubated with medium (shaded histogram), LPS (100 ng/mL), poly I:C (PIC; 0.1 μg/mL), A549-CM, DU-CM, PIC-A549 CM, and PIC-DU CM alone or simultaneously with LPS (continuous line) for 48 h. Then, the expression of CD86 and CD40 was analyzed in CD11c$^+$ cells by flow cytometry. Results shown are representative of three independent experiments performed. The vertical dotted line indicates the peak of the histograms corresponding to the mean fluorescence intensity (MFI) observed in immature DCs (shaded histograms). In the case of CD86 expression, this dotted line shows the peak of MFI of the population of CD11c$^+$ cells expressing higher levels of CD86. MFI are indicated. (B) CD86 and CD40 expression are given as fold-change in MFI compared with that of nonstimulated cells (MoDCs + medium). Results are shown as mean +SEM of at least four different donors from one experiment representative of four experiments performed. (C) MoDCs were cultured as described above with or without an anti-IFN-β blocking antibody (1.5 μg/mL). Then, the expression of CD40 and CD86 was evaluated by flow cytometry at 48 h. MFI is indicated in the figures. Results shown are representative of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, Tukey post test to ANOVA analysis.

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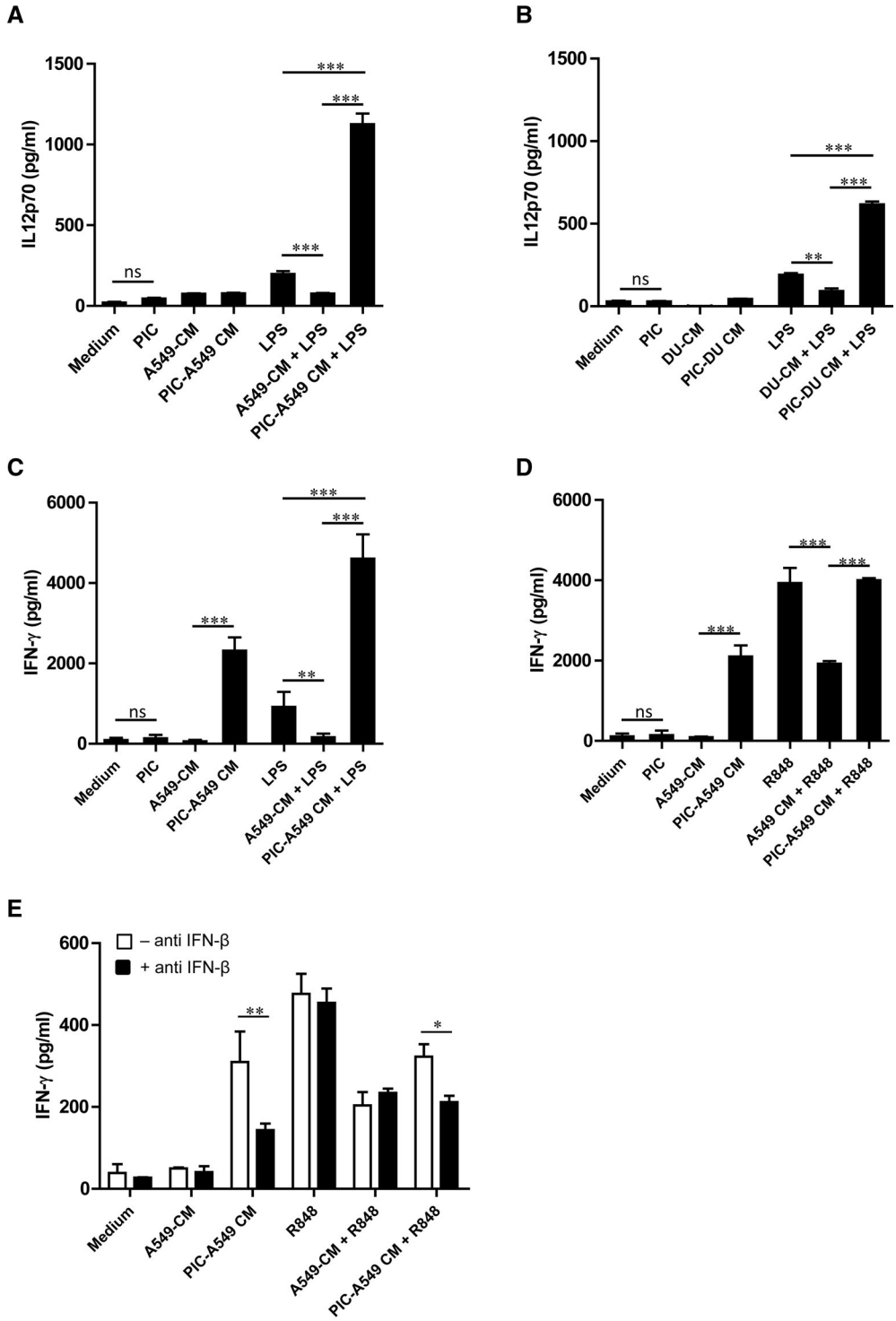

Figure 4. IFN-β produced from poly I:C-treated tumor cells synergizes with TLR ligand to induce IL-12p70 and promote IFN-γ production in an MLR assay. (A, B) IL-12p70 levels evaluated by ELISA in culture supernatants from MoDCs matured with LPS in the presence of CM or PIC-CM from (A) A549 cells or (B) DU145 cells for 24 h. Results are shown as mean +SEM of triplicate wells from one experiment representative of three experiments performed. (C, D) MLR was performed by coculturing allogeneic PBMCs with MoDCs previously matured in the presence of A549-CM, DU-CM, PIC-A549 CM, and PIC-DU CM alone or simultaneously with (C) LPS or (D) R848. IFN-γ release was detected 24 h later in MLR supernatants by ELISA. Data are shown as mean +SEM of two replicates and are from one experiment representative of three experiments performed. (E) Allogeneic PBMCs were stimulated with MoDCs previously matured with R848 in the presence of A549-CM or PIC-A549 CM with or without an anti-IFN-β blocking antibody (1.5 μg/mL). IFN-γ production was detected 24 h later by ELISA. Results are shown as mean + SEM of three samples and are from one experiment representative of three independent experiments performed.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., nonsignificant, Tukey post test to ANOVA analysis.

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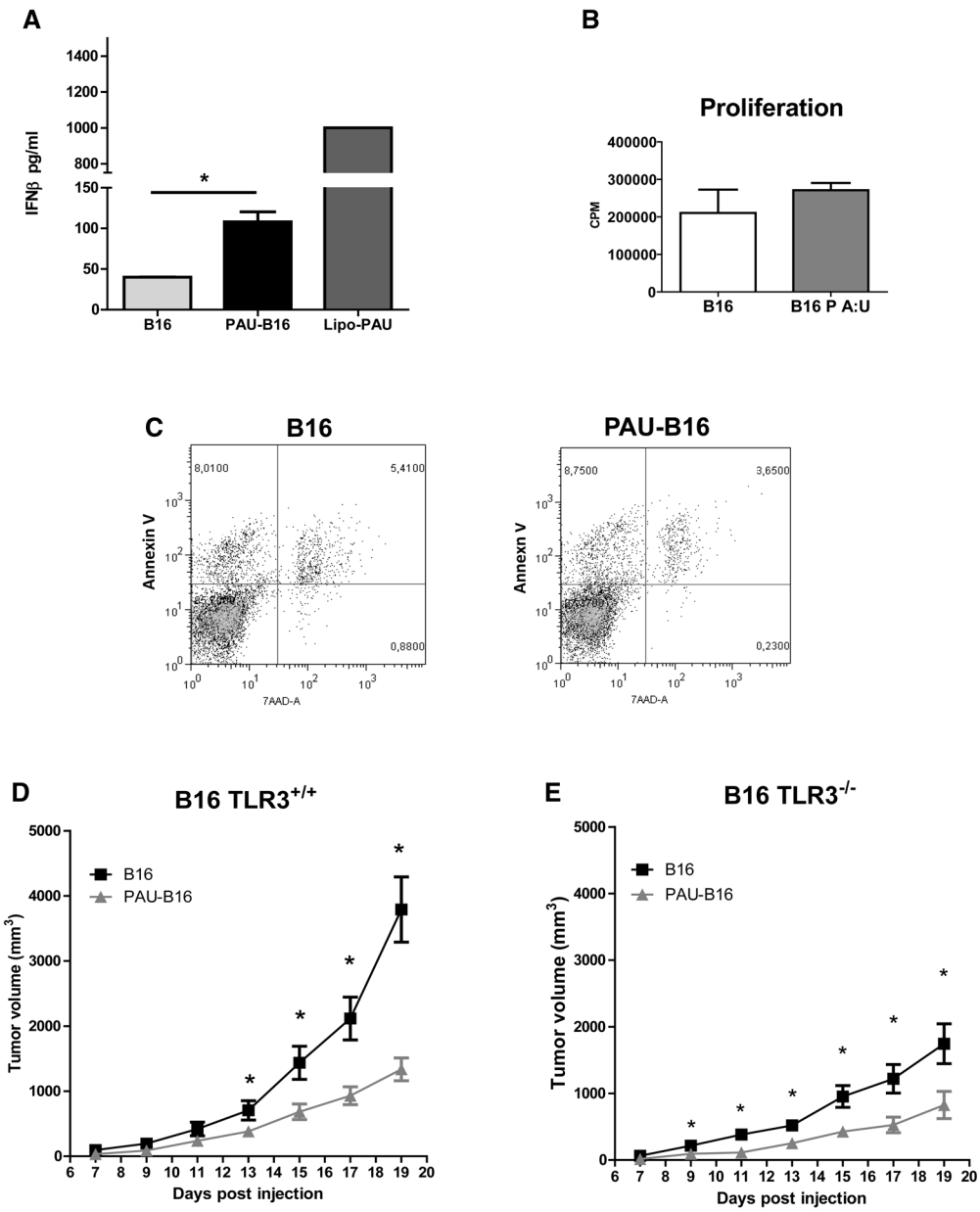

Figure 5. B16 cells activated in vitro with poly A:U induce smaller tumors in TLR3-deficient mice. (A) B16 cells were incubated with PEI alone (B16) or were stimulated with 1 μg/mL of poly A:U complexed to PEI (PAU-B16) or lipofectamine (Lipo-PAU) as described. IFN-β levels were evaluated by ELISA after 24 h. Results are shown as mean ± SD of triplicate wells. $^*p < 0.05$, Tukey post test to ANOVA analysis test. (B) Proliferation and (C) apoptosis levels were measured by ^{3}H-thymidine uptake and by annexin V/7-AAD staining 48 and 24 h, respectively, after stimulation with PEI-PAU. Tumors were induced with B16 cells stimulated in vitro during 24 h with PEI (B16) or PEI-PAU (PAU-B16) in (D) C57BL/6 and (E) TLR3$^{-/-}$ mice. Results are shown as mean ± SEM of ten mice per group and are from one experiment representative of two independent experiments performed. $^*p < 0.05$, Bonferroni post test to two-way ANOVA analysis.

levels were then measured in culture supernantants by ELISA and, as it can be observed in Figure 5A, it showed a two fold increase when poly A:U was used as stimulus. We also tested the ability of B16-CM and PAU-B16 CM to modulate IL-12 secretion. When BMDCs were incubated with CpG in the presence of B16-CM, the secretion of IL-12 was significantly inhibited. However, this inhibitory effect on IL-12 secretion was partially reverted when BMDCs were stimulated with CpG in the presence of PAU-B16 CM (Supporting Information Fig. 1D).

Complexing poly A:U with Lipo-PAU not only generated elevated levels of IFN-β (>1000 pg/mL) but also induced higher levels of apoptosis (data not shown). As it can be seen in Figure 5B and C, poly A:U complexed with PEI neither affected the proliferation rate nor the apoptosis levels of the tumor cells.

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Then, PAU-B16 cells were inoculated into *wt* and TLR3$^{-/-}$ mice. A significant inhibition of tumor growth was observed when tumors were induced by PAU-B16 cells compared to the growth of those induced by nonstimulated cells (B16) (Fig. 5D and E). Since inhibition of tumor growth was observed in both mouse strains (*wt* and TLR3$^{-/-}$), we exclude an effect of remnant poly A:U on APCs from the host and hypothesized that a direct effect of poly A:U on B16 cells was responsible for the inhibition observed. These results indicate that poly A:U signaling on B16 cells induce the production of IFN-β in vitro and that tumors elicited by PAU-B16 cells showed a diminished growth compared to those elicited by nonstimulated cells in both *wt*- and TLR3-deficient mice.

To analyze if type I IFN produced by PAU-B16 could be playing a role in vivo, we inoculated B16 or PAU-B16 cells into mice lacking the IFNAR1 subunit of the type I IFN receptor. Inhibition of tumor growth was observed only in WT mice bearing PAU-B16 tumors (Fig. 6A). Thus, IFN-β signaling is involved in the retardation of tumor growth observed. To explore whether TLR3 on tumor cells play a role in therapeutic settings, we carried out local TLR3 stimulation by treating B16 tumors with PEI-PAU in C57BL/6 and TLR3-deficient mice once tumors became visible (Fig. 6B). In both strains, a significant inhibition of tumor growth was observed; interestingly, the local stimulation of TLR3 present on tumor cells was enough to delay tumor growth in TLR3$^{-/-}$ mice.

Altogether, our results support the hypothesis that type I IFNs produced by poly A:U-stimulated B16 cells, even if secreted in a transient manner, could modify the local environment at the site of tumor cell inoculation, improving DC function and the anti-tumoral immune response, as we had previously reported in a similar experimental model using TLR4 ligands [18, 19].

Discussion

The use of viral dsRNA mimetics in cancer immunotherapy has been explored for several decades [28–35]. They have been assayed with moderate success in different therapeutic settings to treat colorectal carcinoma [29], melanoma [20], gastric [30], bladder [31], ovarian, and breast cancer [32–34]. Viral dsRNA is normally recognized by TLR3 and RLRs in a cell-type and pathogen-type specific manner. TLR3 has been shown to be expressed on human lung carcinoma cells [35] and in lung epithelial cells [36]. Besides, functional expression of TLR3 has been detected in human prostate cancer cell lines and in murine models of prostate cancer [37–39]. Also, it has been published that TLR3 is intracellularly localized in melanoma cells, where it can deliver proapoptotic and antiproliferative signaling. Poly IC activates the TLR3 pathway leading to suppression of the viability of melanoma cells [20, 40]. The murine melanoma B16 cells have also been reported to respond to poly AU [29]. We chose the human lung carcinoma cell line A549, the human prostate carcinoma cell line DU145, and the murine melanoma cell line B16 because they were all reported to express TLR3 and to respond to dsRNA therapy. However, the fact that the levels of IFN-β induction upon poly I:C or poly A:U stimulation were capable of improving DC function had not been reported before.

dsRNA from engulfed apoptotic infected cells is recognized by TLR3 in endosomes, triggering a MyD88-independent response whereas activation of RLRs by viral dsRNA occurs in cytosol and engages a different set of molecular adaptors [1–3]. However, triggering any of these receptors ends in activation of the transcription factors IRF3 and NF-κB and the production of type I IFNs and pro-inflammatory cytokines. A549 cells and DU145 cells (data not shown) upregulate the expression levels of both TLR3 and RLRs. DU145 and A549 human cancer cells respond to dsRNA analogs, inducing an important IFN response and pro-inflammatory cytokines. Phosphorylation of IRF3 was readily observed as well as phosphorylation of STAT1 24 h after the initial stimulus. The latter indicates that type I IFNs are acting in an autocrine fashion on tumor cells, as previously described [8, 9]. Interestingly, the expression of type I IFN receptor has been shown in different epithelial tumors but not in sarcomas, lymphomas, and endocrine tumors [41]. We cannot exclude the possibility of a heterogeneous expression of IFNAR among the tumor cell population, which could promote an in vivo selection of tumor cells refractory to type I IFN stimulation.

Our results show that IFN-β produced by dsRNA-activated tumor cells can also act in a paracrine fashion, as determined by the presence of pSTAT1 after incubation of MoDCs and BMDCs with dsRNA-CM (Fig. 2 and Supporting Information Fig. 1). PIC-CM by itself was capable of inducing the upregulation of *CXCL10* mRNA, CD40, and CD86 expression levels on MoDCs, but not the secretion of IL-12p70. To document that DCs have matured in the critical functional sense and not only by altering their cell surface phenotype, an MLR assay was performed. Interestingly, MoDC that was incubated with PIC-CM prior to coculturing them with allogeneic PBMC generated a highly increased release of IFN-γ in MLR culture supernatants. Both changes in MoDCs, i.e. upregulation of CD40, CD86, and increased MLR stimulation, were abrogated by blocking IFN-β. Surprisingly, MoDC incubated with PIC-CM did not induce IL-12p70 secretion; however, previous data showed that under certain conditions, IL-12p70 can be dispensable for IFN-γ induction. Indeed, in some virus infections, the lack of IL-12 has little or no effect on the induction of Th1 immunity and systemic production of IL-12p70 could not be detected after in vivo administration of poly I:C, whereas poly I:C was superior at inducing systemic type I IFNs and Th1 immune response [42–45]. Murine BMDCs also secreted higher levels of IL-12p70 when they were matured in the presence of PAU-B16 CM. Therefore, a novel aspect of the use of dsRNA mimetics in cancer immunotherapy can be assumed: when tumor cells are activated with dsRNA ligands, they secrete IFN-β at levels that are capable of improving the maturation state and function of DCs, promoting a Th1 response that could be independent of the induction of IL-12.

Tumor-derived factors significantly alter the generation of DCs from hematopoietic progenitors, increase the accumulation of myeloid suppressor cells, and inhibit DCs maturation [22, 23]. When MoDCs were matured with different TLR ligands in the

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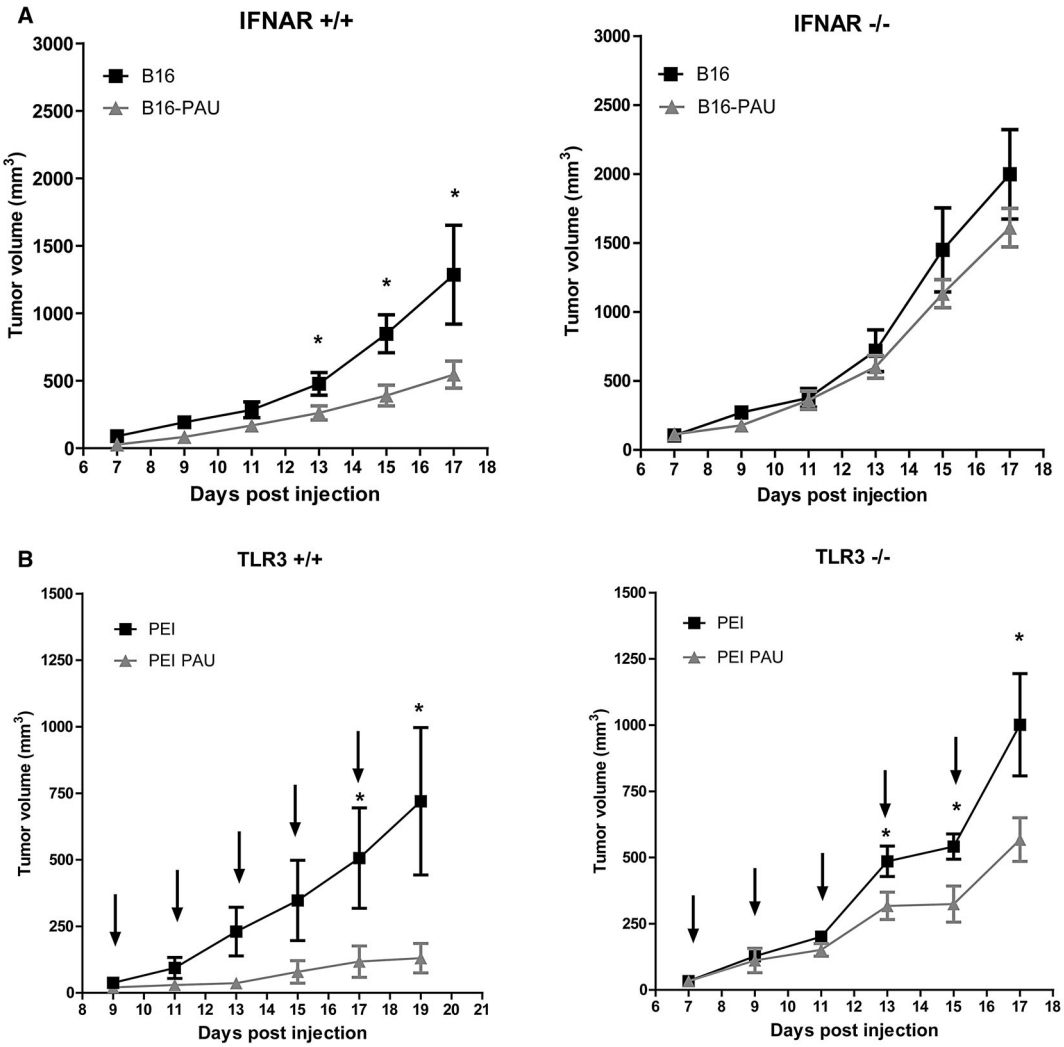

Figure 6. IFN-β produced by dsRNA- activated B16 melanoma cells is involved in tumor growth inhibition. (A) B16 or PAU-B16 tumor growth was evaluated in IFNAR$^{+/+}$ and IFNAR$^{-/-}$ mice, respectively. Results are shown as mean ± SEM of ten mice per group and are from one experiment representative of two independent experiments performed. *$p < 0.05$, Bonferroni post test to two-way ANOVA analysis. (B) Local stimulation of TLR3 on tumor cells retards tumor growth in TLR3$^{-/-}$ mice. PEI-PAU or PEI was injected in C57BL/6 and TLR3$^{-/-}$ tumor-bearing mice five times every 2 days (arrows) at the tumor site ($n = 10$). Data are shown as mean ± SEM and are from one experiment representative of two independent experiments performed. *$p < 0.05$, Bonferroni post test to two-way ANOVA analysis.

presence of tumor CM, expression of co-stimulatory molecules, secretion of IL-12p70, and induction of IFN-γ in MLR were significantly diminished. In contrast, when the maturation was done in the presence of PIC-CM, all these parameters were improved. Indeed, TLR-induced IL-12p70 secretion by DC has been shown to depend on a type I IFN autocrine–paracrine loop [26]. Thus, the simultaneous presence of IFN-β plus the exogenously added TLR ligand, and/or other factors present in PIC-CM such as HMGB1 or other cytokines, could be producing a synergistic effect on maturing MoDCs that can be readily observed in the enhanced values of secreted IL-12p70 and the better capacity of driving an IFN-γ response in the MLR. Similar results were obtained in our previous work, in which murine prostate adenocarcinoma and melanoma cells (TRAMPC2 and B16, respectively) secrete low but reliably detected levels of IFN-β upon TLR4 activation [19]. These low levels of IFN-β were enough to enhance the expression of co-stimulatory molecules on BMDCs as well as to increase the levels of IL-12 secreted. In addition, the frequency of CD11c^{+} tumor infiltrating cells expressing IL-12 was increased in mice bearing LPS-B16 tumors [19]. Interestingly, when B16 cells are activated with poly A:U in vitro (at concentrations that do not induce their apoptosis), the levels of IFN-β produced were much higher (×7) than those produced by LPS-activated B16 cells. However, the inhibition of tumor growth observed when B16 cells were stimulated in vitro with either poly A:U or LPS was very much the same. Thus, it seems that there is not a direct correlation between IFN-β levels and tumor inhibition. Also, poly A:U-stimulated B16 cells induce smaller tumors than nonstimulated B16 cells in WT and TLR3KO mice. In contrast, lack of inhibition of tumor growth was observed when poly A:U-stimulated B16 cells were inoculated

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into IFNAR1$^{-/-}$ mice. We hypothesize that similarly to what we had previously observed using TLR4 agonists, IFN-β secreted by poly A:U-stimulated B16 cells, could be enough to improve the maturation state of local DCs, promoting a more efficient antitumoral response. It has been recently reported that endogenously produced type I IFNs exert an early role in the spontaneous antitumor response, mainly enhancing the capacity of CD8α$^+$ DCs to cross present antigen to CD8$^+$ T cells [14,17]. Indeed, mice lacking IFNAR1 receptor only on DCs cannot reject highly immunogenic tumor. In contrast, mice depleted of NK cells or mice that lack IFNAR1 in granulocytes and macrophage populations reject these tumors normally [14, 17]. Our in vitro and in vivo results allow us to hypothesize that at early moments of tumor implantation, IFN-β produced by dsRNA-stimulated tumor cells could also participate in enhancing the capacity of DCs (more probably CD8α$^+$ DCs) to improve the antitumoral immune response and control tumor growth.

Initially, TLR3 was thought to be expressed mainly by DCs [1–3], so the rational under dsRNA-based therapies was to achieve activation of innate immunity, promoting cross-presentation and triggering a strong Th1 response against the tumor. Later on, TLR3 was shown to be expressed by a broad array of epithelial cells and cancer cells. Stimulating TLR3 on cancer cells with dsRNA was shown to efficiently induce apoptosis. Type I IFN signaling was required for TLR3- triggered cytotoxicity although it was insufficient to induce cell death by itself. On the other hand, dsRNA analogs can also stimulate endothelial cell precursors, inhibiting cell cycle progression and proliferation. Stimulation of TLR3 in cultured endothelial progenitor cells led to increased formation of reactive oxygen species, increased apoptosis, and reduced migration [46].

Our results show that stimulating TLR3 on cancer cells could actually happen in more realistic scenarios such as therapeutic settings in which the dsRNA mimetic is administered once tumors are visible. It has to be highlighted that even in the absence of TLR3 on innate immune cells or on endothelial cells from the host, tumor growth is controlled by the PEI-PAU treatment in a context in which it can only be recognized by tumor cells.

dsRNA mimetics have been proposed to function as multifunctional adjuvants that are able to directly kill the tumor, enhance the host's antitumoral immune response, and control angiogenesis [47–50]. We propose a new function for dsRNA compounds: they can directly act on cancer cells to induce endogenous IFN-β production and contribute to the antitumoral immune response.

Materials and methods

Reagents

LPS from *Escherichia coli* 055:B5 was from Sigma Aldrich. Poly A:U, poly I:C (low molecular weight), or R848 were from InvivoGen. Neutralizing experiments were done using a blocking IFN-β antibody and human soluble recombinant TLR3 (Preprotech). The cationic polymer PEI (cat N 23966) was purchased from Polysciences. The human recombinant IFN-β used as a standard was from Peprotech.

Cell culture

The human lung carcinoma cell line A549, the prostate carcinoma cell line DU145, and melanoma cell line B16 were obtained from ATCC and authenticated by isoenzymology and/or the Cytochrome C subunit I PCR assay. They were periodically cultured in our laboratory for the last 10 and 5 years, respectively. All cell lines were free of *Mycoplasma* infection tested by PCR every 6 months. A549 and DU145 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies).

Stimulation with poly A:U or poly I:C by transfection

We complexed poly A:U to polyethylenimine (PEI-PAU) and poly A:U or poly I:C to Lipo-2000 (Invitrogen) (Lipo-PAU, Lipo-PIC) to enhance its intracellular uptake [51]. A549 and DU145 cells were stimulated with Lipo-PIC (0.1 μg/mL) and B16 cells were stimulated with PEI-PAU (PAU-B16) or Lipo-PAU (1 μg/mL). For stimulation purposes, complexes were added to the cells under serum-free conditions. Control cells were exposed to Lipo-2000 or PEI in the absence of nucleic acids. After 4 h of culture, cells were washed twice with PBS and fresh culture medium was added. Addition of Lipo-2000 or PEI to the cells was considered the initial time of incubation (time 0). To obtain the CM, cells were seeded at 2×10^6 cells/100-mm dish and cultivated for 24 h with culture medium. Then, cells were cultured with Lipo-PIC for 4 h, washed three times with PBS, and incubated for an additional 20 h. Culture supernatants were then harvested and filtered through a 0.22 μm membrane (PIC-CM). Nonstimulated or Lipo-2000-stimulated cell culture supernatants were also collected (CM).

Quantitative reverse transcriptase PCR

RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and reverse transcriptase (Promega) following standard protocols. cDNA samples were then amplified in SYBER green universal PCR master mix buffer (Applied Biosystems) using gene-specific primers pairs (Sigma) to analyze mRNA levels for *TLR3*, *RIG-1*, *MDA5*, *IFNb1*, *CXCL10*, *TNF*, and *IL1b*. cDNA samples were amplified in triplicate with a 7500 Real-Time PCR System (Applied Biosystems) [52]. For each sample, mRNA abundance was normalized to the amount of β-actin and is presented in arbitrary units.

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HEK-Blue IFN-α/β reporter cells assays

The presence of type I IFN in the CMs were evaluated using the HEK IFN-α/β reporter cell system (Invivogen) following the manufacturer's instructions. Briefly, bioactive IFN-α or IFN-β in the sample activates the JAK/STAT/ISGF3 pathway in HEK-Blue cells, subsequently inducing the secretion of secreted embryonic alkaline phosphatase that can be easily determined by a colorimetric assay.

Isolation of CD14$^+$ monocytes and differentiation of MoDCs

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat by Ficoll-Hypaque gradient (GE Healthcare BioSciences) from healthy consenting donors. CD14$^+$ monocytes were purified using CD14$^+$ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec), according to the manufacturer's protocol. Immature MoDCs were generated by culturing CD14$^+$ monocytes in RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen), 800 U/mL GM-CSF, and 500 U/mL IL-4 (BD Biosciences) for 5 days, obtaining more than 90% CD11c$^+$ cells. Medium was replaced with on day 3. For maturation, MoDCs were stimulated with LPS (100 ng/mL), R848 (10 μM), or poly I:C (0.1 μg/mL).

Western blot

Total lysates with intracellular proteins were obtained by treatment of cells with lysis buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Proteins were separated on 10% SDS-PAGE gels and transferred onto a Hybond-C Extramembrane (GE Healthcare). Phospho-IRF3 (Ser396), phospho-STAT1 (Tyr701), and STAT1 were detected by primary rabbit polyclonal antibodies (Cell Signaling). Detection was achieved by HRP labeled secondary antibodies (Cell Signaling) and a chemoluminescence detection kit (GE Healthcare) according to the manufacturer's instructions.

Mixed leukocyte reaction

The allostimulatory capacity of the MoDCs was tested in a MLR. Allogeneic PBMCs were cocultured with differently matured DCs in a 96-well tissue culture microplate and the proliferative response was assessed at various MoDC:PBMC cell ratios after 5 days by measuring thymidine incorporation (1 μCi/mL (methyl-3H)thymidine; specific activity, 50 Ci/mmol; New England Nuclear). Supernatants from MoDC:PBMC cells coculture (ratio 1:10) were harvested at 24 h and analyzed for IFN-γ release by ELISA (eBioscience).

ELISA

Cytokine levels in the culture supernatants were evaluated using ELISA kits for IL-12p70 (BD Biosciences) and IFN-γ (eBioscience) according to the manufacturer's protocol. IFN-β levels were measured in B16 supernatants (PBL Interferon Source) according to the manufacturer's protocol.

Flow cytometry

Anti-CD86 and anti-CD40 mAbs conjugated with their respective fluorochromes were from BD Biosciences. Cytometry was performed in a FacsCanto II flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star Inc.).

Apoptosis assays

B16 cell apoptosis was evaluated by a double-staining procedure with the PE Annexin V binding assay and 7-amino-actinomycin D (7-AAD) staining (BD Biosciences) by flow cytometry. For the gated cells, the percentages of annexin V-negative or annexin V-positive cells and 7-AAD-negative or 7-AAD-positive cells, as well as double-positive cells, were evaluated based on quadrants determined from single-stained and unstained control samples.

Proliferation assay

To evaluate cell proliferation, B16 cells were stimulated with PEI or PEI-PAU for 48 h. Then, cultures were pulsed with 1 μCi/mL (methyl-3H)thymidine (New England Nuclear) for the last 18 h. Results are expressed as cpm ± SD of triplicate determinations.

In vivo tumor challenge

A total of 60 μg of PEI with or without 8 μg of poly A:U (PEI-PAU) was incubated 30 min to form the complexes. B16 melanoma cell line was stimulated with PEI or PEI-PAU for 4 h, washed three times with PBS, and incubated for additional 20 h with complete medium. B16 cells were washed and melanomas were established in C57BL/6, TLR3$^{-/-}$, and IFNAR1$^{-/-}$ mice by subcutaneous injection of 1×10^6 cells into the right flank. Tumor development was monitored every day as described previously (18). To evaluate the therapeutic activity of PEI and PEI-PAU, C57BL/6 and TLR3$^{-/-}$ mice were inoculated with 1×10^6 B16 cells. Once tumors reached approximately 5 mm^3, they were treated intratumorally with PEI (40 μg/200 μL) or with PEI-PAU (40 and 50 μg, respectively, in 200 μL) five times for every 2 days.

Statistics

Statistical analysis was done using the Tukey post test to ANOVA analysis with the InfoStat software (National University of Córdoba). Values of $p < 0.05$ were considered significant.

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Abbreviations: **7-AAD**: 7-amino-actinomycin D · **BMDC**: bone marrow derived DC · **CM**: conditioned medium · **dsRNA**: double-stranded RNA · **IRF**: interferon regulatory factor · **Lipo**: lipofectamine · **MoDC**: monocyte-derived DC · **PEI**: polyethylenimine · **poly A:U**: PAU · **poly I:C**: PIC · **RLR**: RIG-I-like receptor

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