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IFNβ Produced by TLR4-Activated Tumor Cells Is Involved in Improving the Antitumoral Immune Response

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Abstract

Toll-like receptor (TLR) ligands may be a valuable tool to promote antitumor responses by reinforcing antitumor immunity. In addition to their expression in immune cells, functional TLRs are also expressed by many cancer cells, but their significance has been controversial. In this study, we examined the action of TLR ligands on tumor pathophysiology as a result of direct tumor cell effects. B16 murine melanoma cells were stimulated *in vitro* with a TLR4 ligand (LPS-B16) prior to inoculation into TLR4-deficient mice (*Tlr4* $^{lps\text{-}del}$). Under such conditions, B16 cells yielded smaller tumors than nonstimulated B16 cells. The apoptosis/proliferation balance of the cells was not modified by TLR ligand treatment, nor was this effect compromised in immunocompromised nude mice. Mechanistic investigations revealed that IFNβ was the critical factor produced by TLR4-activated tumor cells in mediating their *in vivo* outgrowth. Transcriptional analysis showed that TLR4 activation on B16 cells induced changes in the expression of type I IFN and type I IFN-related genes. Most importantly, culture supernatants from LPS-B16 cells improved the maturation of bone marrow–derived dendritic cells (BMDC) from TLR4-deficient mice, upregulating the expression of interleukin-12 and costimulatory molecules on those cells. BMDC maturation was blunted by addition of an IFNβ-neutralizing antibody. Moreover, tumor growth inhibition observed in LPS-B16 tumors was abrogated in IFNAR1-deficient mice lacking a functional type I IFN receptor for binding IFN. Together, our findings show that tumor cells can be induced through the TLR4 pathway to produce IFN and positively contribute to the antitumoral immune response. *Cancer Res; 72(3); 592–603. ©2011 AACR.*

Introduction

Toll-like receptors (TLR) recognize molecules derived from pathogens as well as endogenous danger signals possessing similar chemical structures (1, 2). Recently, functional TLRs were found to be expressed in cancer cells, but their significance remains controversial (3–12). Whereas most of the therapeutic strategies using microbial products were designed with the idea of activating TLRs present on innate immune system cells (3–10), clear distinction about the separate contribution of immune and cancer cells to the immune response has yet to be done. Stimulation of TLR4, the main receptor of bacterial lipopolysaccharide (LPS), on tumor cells has been shown to have a positive role in tumorigenesis in *in vivo* (13–15), but mainly in *in vitro* settings (16–19). When tumor expression of TLR4 or the adaptor molecule MyD88 was inhibited in tumor cells themselves, opposite conclusions were observed. This suggests a more complex scenario and that the consequences of TLR4 triggering on tumor cells could depend on the type of tumor, the way TLR4 is activated, if such activation is sustained in time, as it would be in the case of chronic activation by endogenous ligands or if it is the result of an acute process (20–24).

Stimulating MAT-LU cells, a rat prostate adenocarcinoma cell line, with LPS *in vitro*, before inoculation, produced significant inhibition of tumor growth in Copenhagen rats (24, 25). The same effect was observed when B16 melanoma cells were stimulated for 48 hours *in vitro* with LPS or monophosphoryl lipid A prior to its inoculation into syngeneic mice. This *in vivo* inhibition of tumor growth depends exclusively on TLR4 present on tumor cell themselves and not on antigen-presenting cells from the host, as it was not observed in TLR4-deficient mice (*Tlr4*$^{lps\text{-}del}$; ref. 24). When LPS-stimulated B16 or MAT-LU cells were inoculated into nude mice, the growth of tumors elicited did not significantly differ from tumors induced by nonstimulated B16 or MAT-LU cells, indicating that the T-cell compartment was required (24).

These findings prompted us to determine which molecular and cellular mechanisms might be involved in this phenomenon. We have identified IFNβ, produced by TLR4-activated

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tumor cells as an important mediator of these effects. Our work brings in a novel aspect to discuss: under specific conditions and, probably, at specific moments of tumor development, TLR4 triggering on tumor cells could positively help the cross-talk between tumor and immune cells, favoring an antitumoral immune response.

Materials and Methods

Reagents

LPS from *Escherichia coli* 055:B5 and non methylated deoxycytidyl-phosphate-deoxyguanosin (CpG) were from Sigma-Aldrich (St. Louis, MO). Ultra-pure LPS from *E. coli* K12 was from InvivoGen.

Animals

C57BL/6 mice were from UNLP, Argentina. C57BL/10ScNJ mice with a deletion of the *Tlr4* gene (*Tlr4*$^{lps\text{-}del}$) were from the Jackson Laboratories. *Il12p40*$^{-/-}$ mice (26) were provided by Dr. Silvia Di Genaro (UNSL, Argentina). *Ifnar1*$^{-/-}$ and *Il28r*$^{-/-}$ mice were provided by Dr. Claude Libert (Ghent University, Belgium). Animals were maintained at the Animal Resource Facility of the CIBICI-CONICET in accordance with the experimental ethics committee guidelines.

Cell lines

Murine B16-F0 melanoma and TRAMPC2 prostate adenocarcinoma cell lines were obtained from American Type Culture Collection and authenticated by isoenzymology and/or the Cytochrome C subunit I (COI) PCR assay. They were periodically cultured in our laboratory for the last 10 and 5 years, respectively. The MB49 bladder cancer cell line was gently provided by Dr. IC Summerhayes (Lahey Clinic, Burlington, MA) and was used after 5 passages of propagation in supplemented medium. All cell lines were free of *Mycoplasma* infection tested by PCR every 6 months. Cells were stimulated *in vitro* with LPS for 48 hours, washed exhaustively, and inoculated subcutaneously into *Tlr4*$^{lps\text{-}del}$ mice. To generate conditioned medium (CM), cells were kept in 2.5% FBS and stimulated or not with 1 μg/mL Ultrapure LPS (LPS-B16 CM or B16-CM, respectively). After 48 hours, supernatants were collected.

Generation of DCs

Dendritic cells (DC) were obtained from bone marrow of *Tlr4*$^{lps\text{-}del}$ mice as described by Inaba and colleagues (27): more than 80% of harvested cells expressed CD11c. CD11c+ cells (2.5×10^5/mL) were incubated with LPS-B16 CM or with B16-CM and exposed to 10 μg/mL of CpG for 24 hours to analyze cytokine secretion by ELISA. LPS-B16 CM and B16-CM was incubated for 1 hour with an IFNβ-blocking antibody (4×10^2NU/mL; PBL Interferon Source), and then used to stimulate bone marrow–derived DCs (BMDC).

In vivo tumor challenge

Melanomas were established in C57BL/6, *Tlr4*$^{lps\text{-}del}$, *Il12p40*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Il28r*$^{-/-}$ mice by subcutaneous injection of 1×10^6 cells into the right flank. Tumor development was monitored every day as described previously (24). To evaluate the therapeutic activity of LPS, C57BL/6 and *Tlr4*$^{lps\text{-}del}$ mice were inoculated with 1×10^6 B16 or 5×10^5 MB49 cells and, once tumors reached approximately 5 mm^3, they were treated intratumorally with LPS (1 μg/200 μL) or with 200 μL of PBS, every other day for 6 consecutive times.

Obtention of tumor-infiltrating cells

Tumor-infiltrating cells were obtained as previously described (24).

Quantitative reverse transcriptase PCR

B16 cells (1×10^6 cells) were stimulated or not with Ultrapure LPS for 6 and 24 hours. In the case of DCs, they were previously incubated with LPS-B16 CM or B16-CM for 20 hours prior to the addition of CpG for 4 hours. mRNA expression was analyzed with mouse toll-like receptor signaling pathway, RT^2*Profiler* PCR Array, SABiosciences according to the manufacturer's protocol. The PCR array was done in ABI Prism7500 Cycler Detection System. The following primers were used: *IFNb1* Fw5′-TTACACTGCCTTTGCCATCC, Rev5′-ACTGTCTGCTGGTGGAGTTCAT; *IL-6* Fw5′-GAGGATACCACTCCCAACAGACC-3′, Rev5′-AAGTGCATCATCGTTGTTCATACA-3′; *GAPDH* Fw5′-TCACCACCATGGAGAAGGC-3′, Rev5′-GCTAAGCAGTTGGTGGTGCA-3′. To analyze the data the 2(-Delta Delta C(T)) method was used (28).

ELISA

Cytokines were measured by ELISA Kits (e-Biosciences, BD-Bioscience, and PBL Interferon Source) according to the manufacturer's protocol. IFNβ levels were measured in 5-fold concentrated tumor supernatants (Vivaspin sample concentrator; GE Healthcare Life Science).

Flow cytometry

Monoclonal antibodies conjugated with their respective fluorochromes were from BD-Bioscience and e-Biosciences. Intracellular cytokine was detected after stimulating cells for 5 hours with PMA (10 ng/mL) and Ionomycin (1 μg/mL; Sigma-Aldrich). Brefeldin A (10 μg/mL; Sigma) was added for the last 4 hours of cell culture. Results were analyzed using FlowJo software (Tree Star, Inc.).

Statistics

Statistical analysis was done using the Tukey posttest to ANOVA analysis with the InfoStat software (National University of Córdoba). Values of P less than 0.05 were considered significant.

Results

Triggering TLR4 on B16 cells induces the expression of important modulators of DC activity

To analyze the expression of genes related to TLR4-mediated signal transduction and effector molecules by a qPCR array, B16 cells were either nonstimulated or stimulated with 1 μg/mL LPS for 6 and 24 hours. Distinctive TLR4 activation of downstream genes in B16 cells is shown in the gene expression heat map in Fig. 1A. Whereas more than a 6-fold induction was

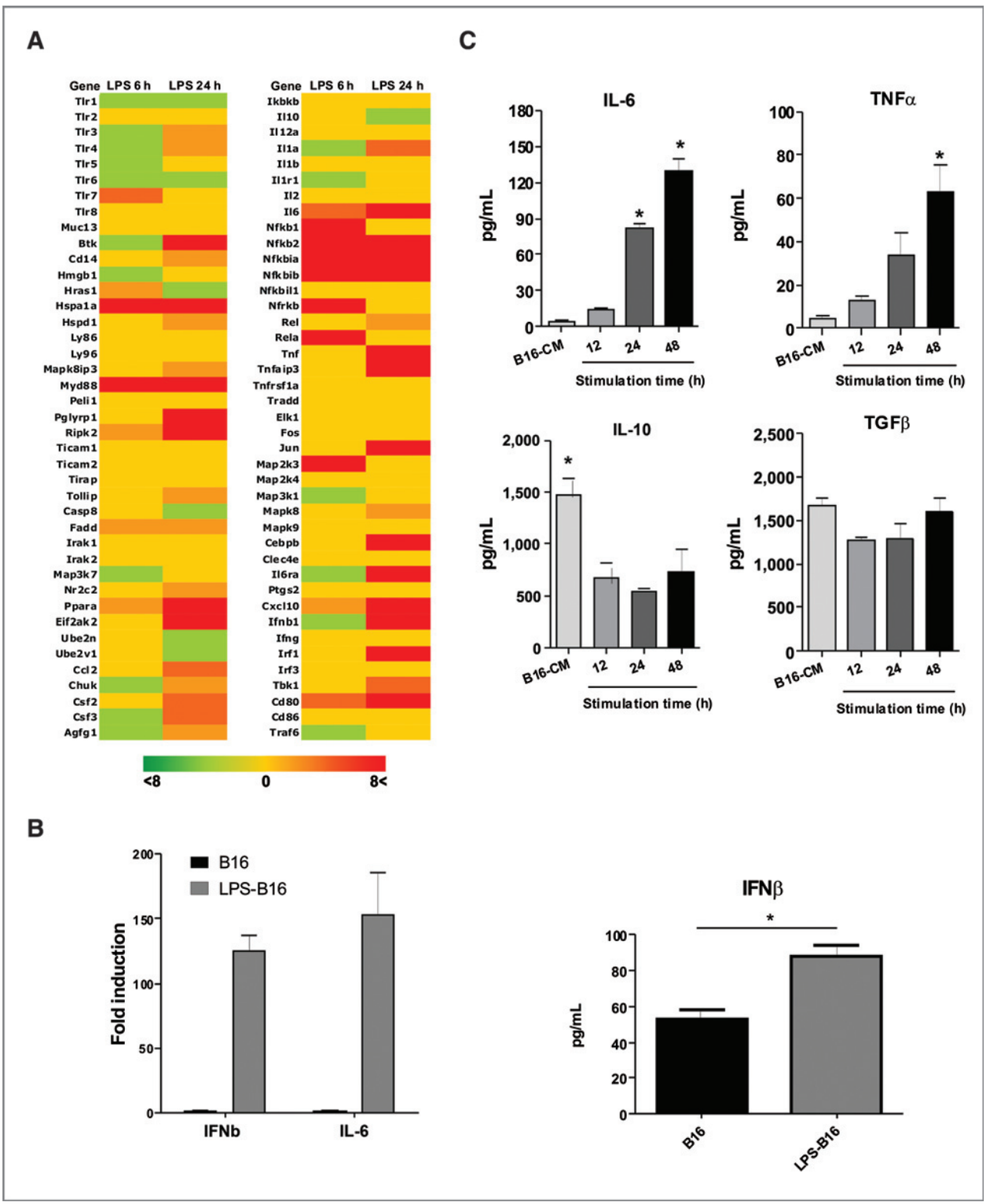


Figure 1. Triggering TLR4 on B16 cells induces the expression of important modulators of DC activity. A, heat map of qPCR array analysis. Gene expression was compared with the expression in nonstimulated B16 cells. B, *IFNb1* and *IL-6* mRNA expression in B16 or LPS-B16 cells normalized to glyceraldehyde-3-phosphate dehydrogenase expression. IFNβ levels in 5-fold concentrated culture supernatants from B16 cells or LPS-stimulated B16 cells (LPS-B16) evaluated by ELISA. Results indicate means ± SD of triplicate wells. *, $P < 0.05$. C, cytokine levels evaluated by ELISA in B16-CM and LPS-B16 cells stimulated at the indicated times. Results indicate mean ± SD of triplicate wells. *, $P < 0.05$.

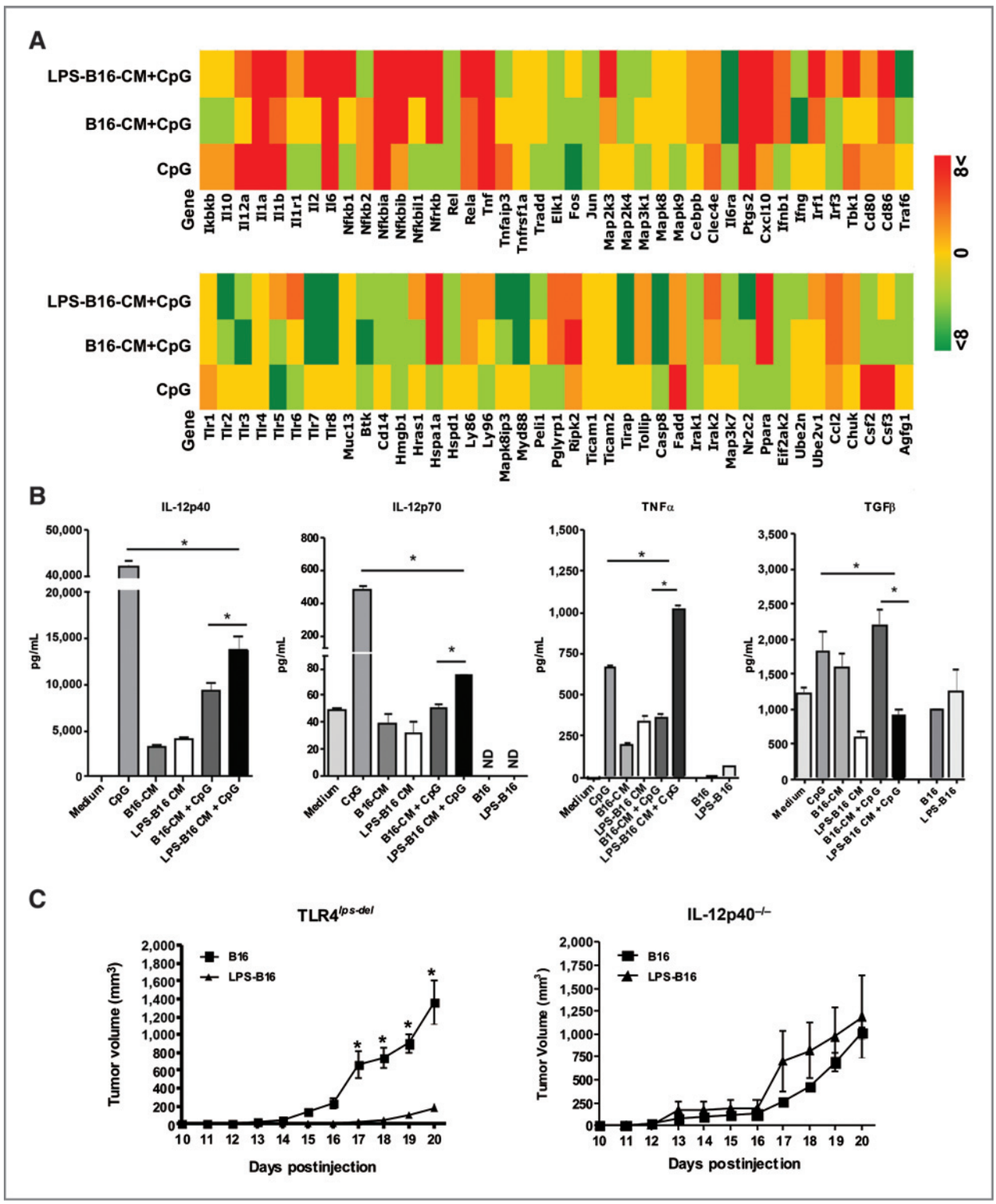
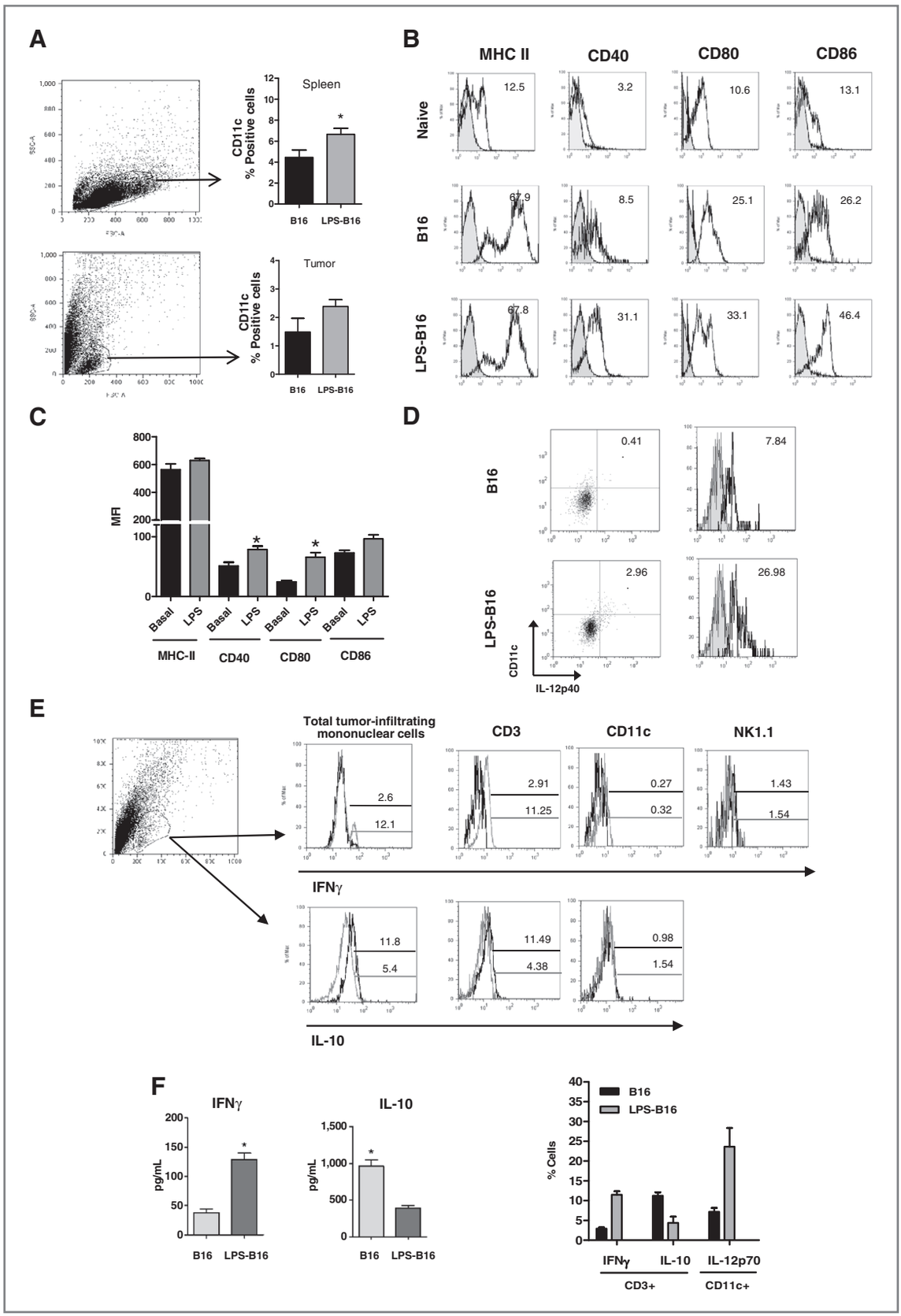


Figure 2. LPS-B16 CM can partially reverse the suppressive effect of B16-CM on TLR4$^{lps\text{-}del}$ BMDC maturation. A, heat map of qPCR array analysis. TLR4$^{lps\text{-}del}$ BMDCs incubated with medium, B16-CM, or LPS-B16 CM for 20 hours and then stimulated with CpG for 4 hours. Gene expression was compared with that from immature TLR4$^{lps\text{-}del}$ BMDCs. B, cytokine levels in culture supernatants of TLR4$^{lps\text{-}del}$ BMDCs under the different stimulating conditions. Cytokines present in B16 and in LPS-B16 supernatants (B16 and LPS-B16) are also shown. Results indicate means ± SD of triplicate wells. ND, not detectable, *, $P < 0.05$. C, tumors were induced with B16 or LPS-B16 cells and their volume was measured ($n = 6$; mean ± SEM). Results are representative of 3 independent experiments. *, $P < 0.05$.



observed for the *NFkB* mediator genes after 6 hours of stimulation (*Nfkb1*, *Nfkb2*, *Nfkbia*, *Nfkbib*, *RelA*, *MyD88*, and others), most of the effector molecules were induced after 24 hours poststimulation (150x *IL-6*, 6x *CCL2*, 6x *Csf2*, 13x *TNFa*). Interestingly, an important increase in the expression of genes from the IRF pathway (160x *Cxcl10*, 11x *Irf1*), mainly *IFN beta* (120x) was observed (Fig. 1A). This upregulation of *IFN beta* expression levels was confirmed by quantitative reverse transcriptase PCR using a different set of primers (Fig. 1B). The upregulation of *IFNbeta* mRNA expression levels was also observed in 2 other murine tumor cell lines expressing TLR4: the MB49 and TRAMPC2 cells (Supplementary Fig. S1A). Both cell lines generated smaller tumors once activated via TLR4, prior to their *in vivo* inoculation in $Tlr4^{lps\text{-}del}$ and C57BL/6 mice (Supplementary Fig. S1B and S1C). In the case of the TRAMPC2 prostate cancer model, the inhibition of tumor growth observed was not statistically significant, but the incidence of tumor-bearing animals was lower in the LPS group (Supplementary Fig. S1C). Low levels of secreted IFNβ could also be reliably measured by ELISA in LPS-activated tumor cells (Fig. 1B and Supplementary Fig. S1A). Interleukin (IL)-6 and TNFα secreted levels increase with time in LPS-stimulated B16 cells supernatants, whereas the levels of IL-10 drop after just 12 hours of stimulation of B16 cells with LPS (Fig. 1C). In contrast, the levels of TGFβ were not modified.

LPS-stimulated B16 cell supernatants can partially reverse the suppressive effect of B16 cell–derived factors on $Tlr4^{lps\text{-}del}$ BMDC maturation

Tumor-derived factors significantly inhibit the generation of DCs from hematopoietic progenitors, increase the accumulation of myeloid suppressor cells (usually characterized as GR1+ CD11b+ cells), and inhibit DCs maturation (29–31). To see if factors secreted by LPS-stimulated B16 cells could somehow overcome this inhibitory effect, we collected supernatants from B16 cells stimulated for 48 hours with 1 μg/mL Ultrapure LPS (LPS-B16 CM) or from nonstimulated cells (B16-CM) and incubated with BMDCs from $Tlr4^{lps\text{-}del}$ mice for 20 hours before inducing their maturation with CpG. We used BMDCs from $Tlr4^{lps\text{-}del}$ mice to exclude any direct effect on the maturation state of the DCs by vestigial LPS in the CM. We hypothesize that factors present in B16-CM would interfere in this process, inhibiting the transcription of key effector molecules, whereas LPS-B16 CM would do it at a lesser extent or would not do it at all. After 4 hours of stimulation of $Tlr4^{lps\text{-}del}$ BMDCs with CpG, transcription of genes coding for effector molecules such as *IL-12a* (x8), *IL-1a* (x12), *IL-1b* (x7), *IL-6* (x11), *TNFa* (x20), *Csf2* (x15), *Csf3* (x16) was increased (Fig. 2A). Transcription of genes related with the NFκB complex was not observed, although the expression of inhibitors of the pathway such as *NFkBia* (IkBalpha) showed a moderate raise (x11). When the same analysis was done in $Tlr4^{lps\text{-}del}$ BMDCs that had been incubated with B16-CM and LPS-B16 CM prior to maturation, some intriguing findings were detected. Transcription of genes coding for some effector molecules was not extremely altered (such as *IL-1a* and *b*, *TNF* and *IL-6*). Other genes were extremely downregulated in both experimental groups subjected to tumor supernatants (such as *Csf2* and *Csf3*). In contrast, differences were found in the expression of certain genes among the 3 experimental groups. When transcription of genes of the *NFkB* complex was evaluated, only $Tlr4^{lps\text{-}del}$ BMDCs matured in the presence of LPS-B16 CM showed a significant increase (x380 *NFkb1*, x468 *Rela*), indicating a more sustained or stronger stimulation of the cells. Reasonably, a strong increase in transcription levels of both inhibitor molecules such as *NFkBia* (IkBalpha) and *NFkBib* (IkBbeta) was also observed (x80 and x1250 compared with x33 and x375 in B16-CM). Interestingly, the expression of *IL-12a* that was inhibited in DCs incubated with B16-CM (x1.8) was partially restored when LPS-B16 CM (x5) was present at the time of maturation.

Next, we examined the ability of B16-CM and LPS-B16 CM to modulate cytokine secretion by DCs. As expected, immature DCs (iDCs) exposed to CpG showed a significant increase in IL-12p40 secretion (Fig. 2B). A 5-fold reduction in IL-12p40 secretion was observed when iDCs were simultaneously incubated with B16-CM and CpG for 24 hours. This reduction was not due to an expansion of Gr1+CD11c+ at the expense of CD11c+ cells, because the exposure of the iDCs for 24 hours to CM did not alter the final phenotype of the cells (Supplementary Fig. S2A and B). In contrast, IL-12p40 secretion levels were partially restored when the cells were incubated with LPS-B16 CM (3-fold reduction, $P < 0.05$; Fig. 2B). Similar results were observed when IL-12p70 levels were evaluated: even though CpG alone is a poor inducer of IL-12p70 (32), detectable levels of IL-12p70 could be reliably measured in $Tlr4^{lps\text{-}del}$ DCs exposed to CpG (Fig. 2B and Supplementary Fig. S2C). These levels were almost abrogated when $Tlr4^{lps\text{-}del}$ DCs were incubated with B16-CM, LPS-B16 CM, and B16-CM plus CpG. In contrast, IL-12p70 was partially restored when $Tlr4^{lps\text{-}del}$ DCs were matured with CpG in the presence of LPS-B16 CM (Fig. 2B and Supplementary Fig. S2C). When TNFα (Fig. 2B) and IL-6 (data not shown) were measured a similar effect was observed. The levels of these cytokines present in B16-CM or

Figure 3. LPS-B16 tumor–bearing $TLR4^{lps\text{-}del}$ mice show DCs with a more mature phenotype. A, percentages of CD11c+ cells in mice injected with B16 or LPS-B16 cells. Results show mean ± SD of 3 independent experiments. *, $P < 0.05$. B, costimulatory molecule expression on spleen CD11c+ cells from naive (without tumor) or B16 or LPS-B16 tumor–bearing mice (continuous line); isotype control (shaded histogram). A representative experiment is shown. C, mean fluorescence intensity (MFI) values of distinct activation markers determined on spleen CD11c+ cells from naive or tumor-bearing mice. Results show mean ± SD of 3 independent experiments *, $P < 0.05$. D, intracellular IL-12p40 staining on tumor-infiltrating CD11c+ cells. Numbers indicate the percentage of IL-12p40+ CD11c+ cells. Shaded histogram, isotype control; continuous line, IL-12p40+CD11c+ cells (right). E, *ex vivo* production of IFNγ and IL-10 by total tumor-infiltrating mononuclear cells, CD3+, CD11c+, and NK1.1+ cells from B16 (black histogram) or LPS-B16 (grey histogram) tumors. A representative experiment is shown. F, IFNγ and IL-10 in culture supernatants of tumor-infiltrating mononuclear cells activated *ex vivo* with PMA–Ionomycin evaluated by ELISA (left). *, $P < 0.05$. Percentages of IFNγ, IL-10 in CD3+cells and IL-12p70 in CD11c+cells in B16 or LPS-B16 tumors (right). Results show mean ± SD of 3 independent experiments.

LPS-B16 CM did not significantly interfere with those secreted by DCs.

TGFβ is also secreted by the tumor itself as an escape mechanism (29, 30, 33). TGFβ levels in B16-CM and LPS-B16 CM were similar to those measured in *Tlr4*$^{lps\text{-}del}$ BMDCs in basal conditions. When *Tlr4*$^{lps\text{-}del}$ BMDCs were incubated with B16-CM for 20 hours, TGFβ levels raised approximately 1.5 times compared with those detected in the B16 cells supernatants, suggesting that under these experimental conditions, DCs are also a source of TGFβ. When BMDCs were incubated with LPS-B16 CM for 20 hours, TGFβ levels dropped significantly (Fig. 2B). Surprisingly, maturation of *Tlr4*$^{lps\text{-}del}$ BMDCs in the presence of LPS-B16 CM significantly reduces the levels of TGFβ secreted, when compared with those secreted by DCs matured in the presence of B16-CM, supporting our hypothesis that DC proactivating factors are increased in LPS-B16 CM.

In vitro stimulation of B16 cells with TLR4 agonists, such as LPS or monophosphoril lipid A, for 48 hours before inoculation produced significant inhibition of tumor growth in syngeneic C57BL/6 or in *Tlr4*$^{lps\text{-}del}$ mice. Because this effect was not observed in athymic nude mice, T cells must somehow be involved. DCs are crucial in initiating a Th1 response, mainly through the secretion of IL-12. To test whether cytokines secreted by DCs were crucial intermediaries in the inhibition of tumor growth observed in LPS-B16 tumor-bearing mice, we induced tumors with B16 or LPS-B16 cells in *Tlr4*$^{lps\text{-}del}$ and IL-12p40–deficient mice. As expected, LPS-B16 tumors were significantly smaller than B16 tumors in TLR4$^{lps\text{-}del}$ mice; in contrast, both experimental groups (B16 and LPS-B16) followed the same pattern of tumor growth in *Il12p40*$^{-/-}$ mice, indicating that either IL-12 or IL-23 (which share the IL-12p40 subunit) is required in the inhibition of tumor growth observed in *wt* animals bearing LPS-B16 tumors (Fig. 2C). This experiment also suggests that DCs, one of the most prolific producers of these cytokines could be involved in the inhibition of tumor growth observed in our *in vivo* model.

LPS-B16 tumor–bearing mice show spleen DCs with a more mature phenotype as well as increased frequencies of IL-12+ CD11c+ and IFNγ+ tumor-infiltrating cells

Then, *Tlr4*$^{lps\text{-}del}$ mice were subcutaneously injected with B16 or LPS-B16 cells, sacrificed on day 20 and CD11c+ and GR1+ CD11b+ cells from spleens and tumors were analyzed by flow cytometry. A higher percentage of CD11c+ cells and a lower percentage of GR1+ CD11b+ cells were found in the spleens of animals bearing LPS-B16 tumors, compared with those bearing B16 tumors or normal control (Fig. 3A and Supplementary Fig. S3). Spleen CD11c+ cells from all tumor-bearing animals showed an activated phenotype, with a higher percentage of cells expressing increased levels of MHC class II and costimulatory molecules (Fig. 3B). However, a further enhancement in the expression levels of CD40, CD80 and, in some experiments, of CD86 was observed in CD11c+ cells from animals bearing LPS-B16 tumors (Fig. 3B and C).

Intratumoral infiltrating mononuclear cells were also analyzed. Although the percentage of CD11c+ cells in LPS-B16 tumors did not change (Fig. 3A), the frequency of IL-12+ CD11c+ cells (Fig. 3D) was increased. Moreover, when tumor-infiltrating mononuclear cells were stimulated *ex vivo* with PMA–ionomycin for 5 hours and IFNγ–IL-10 cytokine intracellular staining was done, an increased frequency of IFNγ+ CD3+ cells was observed in LPS-B16 tumors compared with B16 tumors. The opposite was observed when IL-10+ CD3+ cells were analyzed (Fig. 3E and F).

Thus, LPS-B16 cells inoculated into *Tlr4*$^{lps\text{-}del}$ mice promoted a better functionality of DCs *in vivo*, which could be associated with a more efficient Th1 response *in situ* and the reduced tumor growth observed.

IFNβ produced by TLR4-activated tumor cells is involved in reversing the suppressive effect of B16 cell–derived factors on *Tlr4*$^{lps\text{-}del}$ BMDC maturation

Induction of type I IFN during stimulation of DCs through innate receptors is essential for optimal production of the IL-12 p70 heterodimer (33). Thus, we investigated the putative role of IFNβ present in LPS-B16 CM in restoring the suppressive effect of B16-CM on *Tlr4*$^{lps\text{-}del}$ BMDC maturation. We looked at CD40 expression in *Tlr4*$^{lps\text{-}del}$ BMDCs incubated with LPS-B16 or B16-CMs for 20 hours and matured with CpG. A neutralizing anti-IFNβ was added to the CMs 1 hour before incubating them with DCs. As expected, the percentage of DCs expressing CD40 increases to an 80% after 4 hours of stimulation with CpG (Fig. 4A). This increase is not affected with the addition of anti-IFNβ to the culture medium. An inhibition in the percentage of CD40+ CD11c+ cells and in the levels of CD40 expression is observed when *Tlr4*$^{lps\text{-}del}$ BMDCs were incubated with B16-CM and then matured with CpG, which is restored when the cells are matured in the presence of LPS-B16 CM. This restitution of CD40 expression was abrogated when the neutralizing anti-IFNβ was added, indicating that IFNβ is participating in the improvement of DC maturation observed (Fig. 4A and Supplementary Fig. S4). Similar results were obtained when IL-12p70 secreted by the DCs under the different conditions were measured by ELISA (Fig. 4B).

To confirm the role that type I IFN could be playing in our model, we inoculated B16 or LPS-B16 cells into mice lacking the IFNAR1 subunit of the type I IFN receptor. Inhibition of tumor growth was observed only in wild-type mice bearing LPS-B16 tumors, indicating that type I IFN signaling is involved in tumor growth inhibition induced by activation of TLR4 expressed on tumor cells (Fig. 4C). To observe whether IL-28 (a distinct category of type I–like IFN, also referred to as type III IFN; ref. 34) could be playing a role in tumor inhibition in our model, we inoculated B16 cells stimulated or not with LPS in *Il28r* KO mice. Inhibition of tumor growth was observed in wild-type mice, but only a partial and nonstatistically significant inhibition of tumor growth was seen in the *Il28r* KO mice, indicating that type III IFNs could also be playing a role in the phenomenon (Fig. 4D).

Local stimulation of TLR4 present on tumor cells retards temporally tumor growth in TLR4-deficient mice

To investigate whether TLR4 on tumor cells play a role in therapeutic settings, we carried out local TLR4 stimulation by

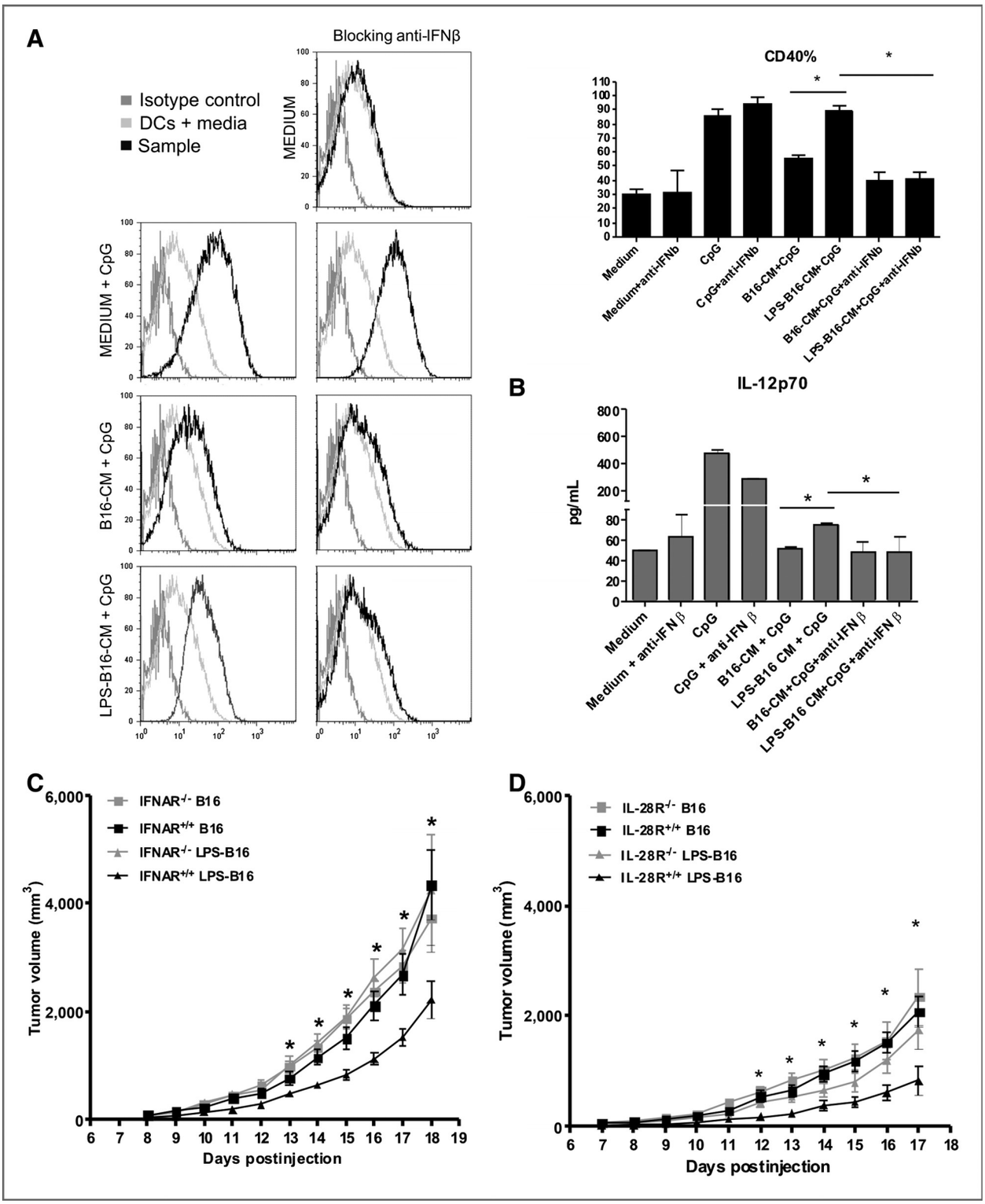


Figure 4. IFNβ derived from LPS-stimulated B16 cells induce TLR4$^{lps\text{-}del}$ dendritic cell activation. A, representative CD40 expression dot plot (left) and percentages of CD40+ cells (right) in CD11c+ BMDCs cultured with or without an anti-IFNβ–blocking antibody. B, IL-12p70 levels in CD11c+ BMDCs cultured as in Fig. 2B with or without an anti-IFNβ–blocking antibody. Results show mean ± SD of triplicate experiments. C, tumors were induced with B16 or LPS-B16 in IFNAR$^{-/-}$ mice ($n = 9$). Results are representative of 2 independent experiments. *, $P < 0.05$ for IFNAR$^{+/+}$ LPS-B16 versus IFNAR$^{-/-}$ LPS-B16, IFNAR$^{+/+}$ B16, and IFNAR$^{-/-}$B16 (mean ± SEM). D, tumors were induced with B16 or LPS-B16 in IL-28R$^{-/-}$ mice ($n = 10$). *, $P < 0.05$ for IL-28R$^{+/+}$ LPS-B16 versus IL-28R$^{+/+}$ B16 and IL-28R$^{-/-}$ B16 (mean ± SEM).

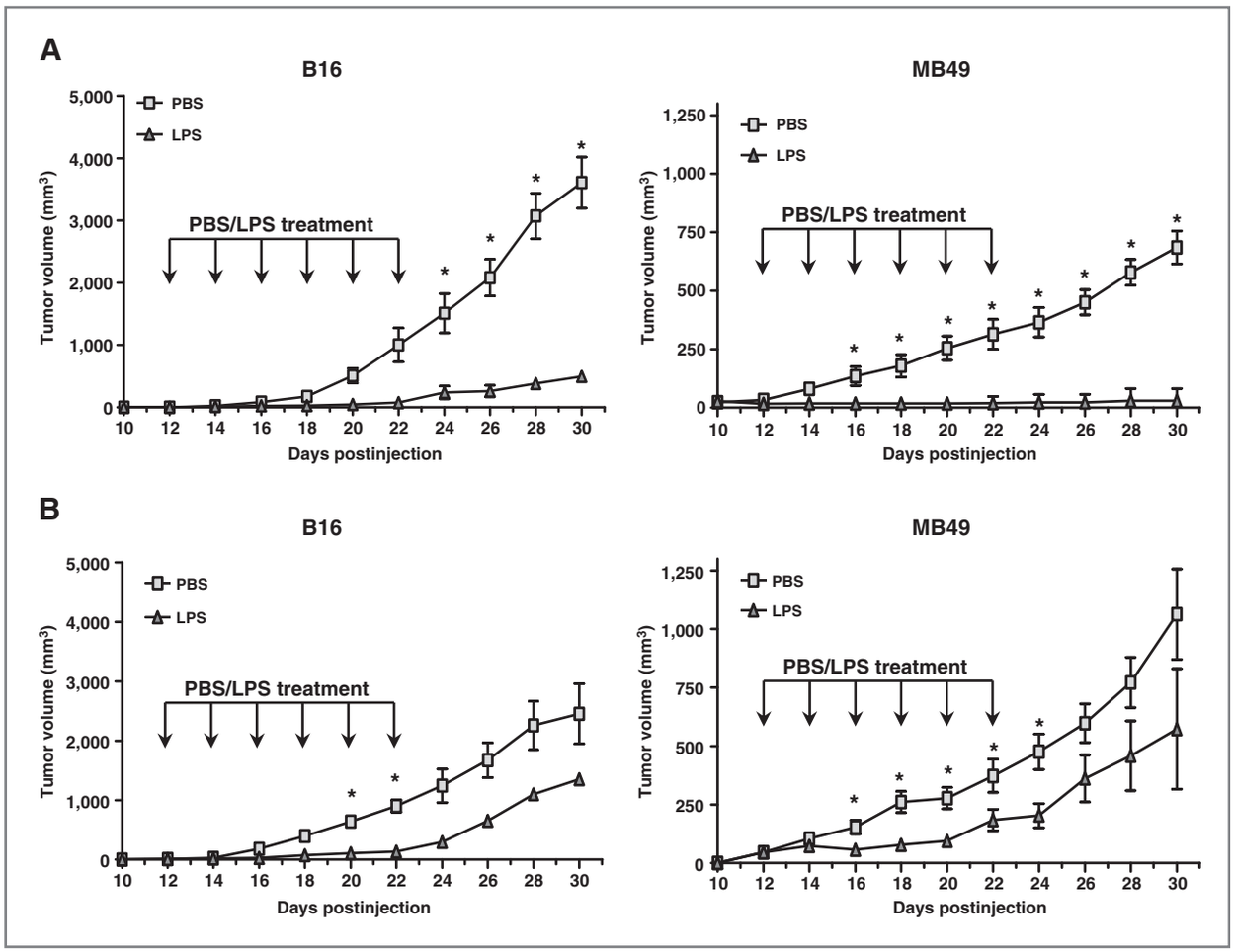


Figure 5. Local stimulation of TLR4 present on tumor cells retards temporally tumor growth in TLR4$^{lps\text{-}del}$ mice. LPS or PBS was injected in C57BL/6 (A) and TLR4$^{lps\text{-}del}$ (B) tumor-bearing mice for 6 days (arrows) at the tumor site ($n = 8$). *, $P < 0.05$.

injecting LPS intratumorally in B16 and MB49 tumors in C57BL/6 and *Tlr4*$^{lps\text{-}del}$ mice. In both models, a significant inhibition of tumor growth was observed in C57BL/6 animals that received LPS injections once tumors became visible (Fig. 5A). Interestingly, B16 and MB49 tumors in *Tlr4*$^{lps\text{-}del}$ mice also showed a significant but transient inhibition of tumor growth that was sustained as long as the LPS treatment was maintained. Immediately after the LPS treatment was finished, tumors began to grow (Fig. 5B). However, although nonstatistically different, tumor volumes kept being slightly smaller in LPS-treated mice.

Discussion

B16-derived soluble factors strongly suppressed the activation of BMDCs in response to CpG, downregulating the secretion levels of IL-12p40, IL-12, and TNFα and diminishing the percentage of CD40+ CD11c+cells. Interestingly, when BMDCs from *Tlr4*$^{lps\text{-}del}$ mice were matured with CpG in the presence of LPS-B16 CM, the inhibition observed in the different parameters was partially reversed. Although DCs obtained from spleens of B16 and LPS-B16 tumor–bearing mice both show an activated phenotype, it is improved in the latter group. Also, an increase in IL-12+ CD11c+ cells infiltrating tumors was observed, further providing evidence of their improved functional state *in vivo*. Initial CD4+ T-cell activation occurs in secondary lymph nodes, but to elicit effector functions, in lymph nodes or in nonlymphoid tissues such as tumors, they need to see again the specific MHC II–peptide complexes to produce effector cytokines, such as IFNγ (35). Tumor cells do not express MHC class II molecules; therefore, infiltrating DCs would sustain CD4 T-cell stimulation (36, 37). Tumor-infiltrating DCs have already been described in B16 melanomas, in numbers even higher than those found in normal skin, being poor producers of IL-12. The fact that tumor-infiltrating mononuclear cells produce higher levels of IFNγ and reduced levels of IL-10, argues in favor of a more efficient immune response taking place in LPS-B16 tumor–bearing animals, responsible for controlling tumor growth.

Thus, upon TLR4 activation, B16 cells could secrete a different pattern of soluble factors and cytokines, which would

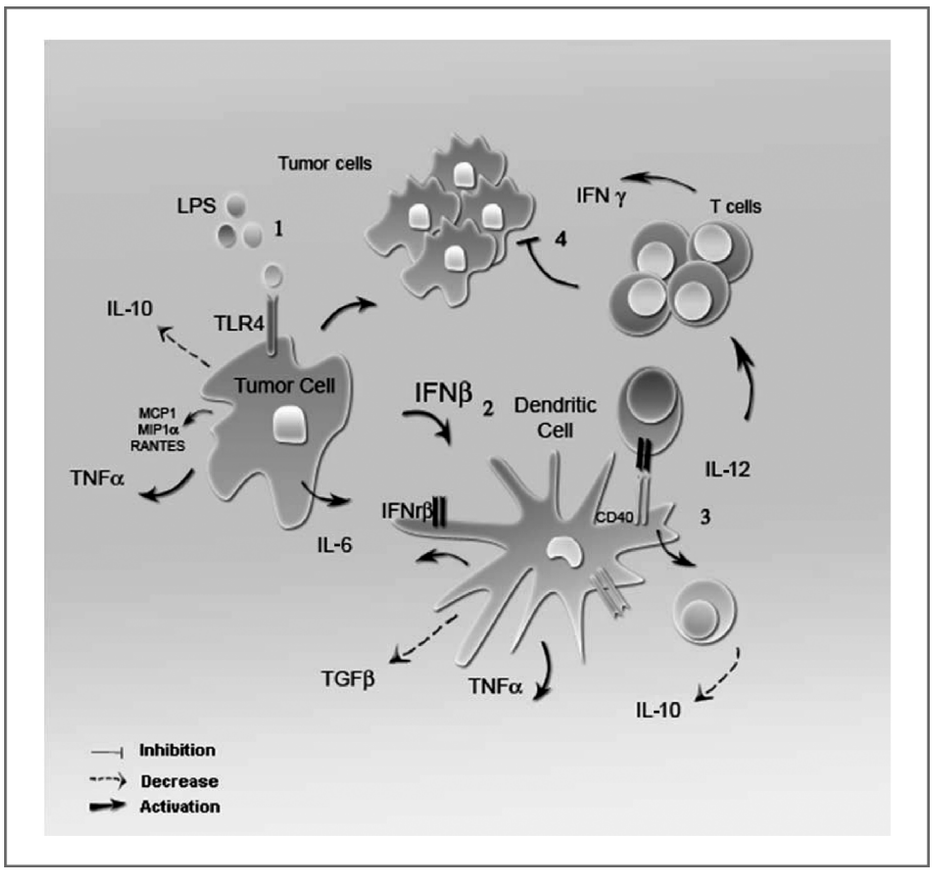


Figure 6. Proposed model (1) LPS-B16 cells secrete IFNβ and other modulators of DC activity; (2) IFNβ can significantly restore the IL-12–secreting capacity of DCs at the site of inoculation, improving their maturation state; (3) DCs promote a Th1 response against the growing tumor (4).

favor the balance to a friendlier environment for activating DCs. Transcriptional analysis of LPS-stimulated B16 cells confirms this idea. The expression of *GM-CSF* and *G-CSF* genes considerably increases as well as those involved in the type I IFN pathway. Also, the levels of IL-6 and TNFα are increased in LPS-stimulated B16 cell supernatants. IL-6 has been involved in activation of STAT3 in DCs, which in turn would contribute to the inhibition of their phenotypic and functional maturation (38). In contrast, type I IFN, TNFα, and granulocyte macrophage colony-stimulating factor (GM-CSF) have potent effect in inducing maturation of DCs and have been used as adjuvant therapy for advanced-stage melanoma in patients and in mice (39). Thus, cytokines reported to have modulatory effects on DC activation could simultaneously be present at augmented or diminished levels in LPS-B16 CM making a cocktail that would provide signals to DCs that, in turn, will be differentially integrated.

The involvement of DCs in our tumor model could also be indirectly analyzed by the lack of inhibition of tumor growth induced by inoculation of LPS-stimulated B16 cells in mice deficient for IL-12p40 subunit, arguing that antigen-presenting cells, main producers of IL-12 and IL-23 and presumably DCs, play a key role in our model. Both IL-12p40 and IL-12p70 secretion levels are partially restored when TLR4$^{-/-}$ DCs are matured with CpG in the presence of LPS-B16 CM. Type I IFNs was an obvious candidate to be playing a role in our model because type I IFNs play a crucial role in the induction of IL-12p70 (40, 41). Type I IFNs have an established role in regulating the innate and adaptive arms of the immune system (34, 42–44), and when given exogenously to tumor patients, retard tumor growth and inhibit angiogenesis (34). Mice challenged with tumor cells that produce type I IFN as recombinant protein do not develop tumors (45–47). B16 tumors grew faster in *Ifnb1*$^{-/-}$ mice and they reach larger sizes and higher weights compared with *wt* mice (45–47). Also, *Ifnar1*$^{-/-}$ mice are more susceptible to the development of sarcomas induced with a chemical carcinogen. As it has been reported previously, the levels of IFNβ secreted under our experimental conditions were very low and difficult to measure with commercial ELISA kits (45, 46). U'Ren L and colleagues have found that *in vitro* cultured tumor tissues spontaneously released low concentrations of IFN-α (in the range of 5 pg/mL) and that concentrations of IFN-β in cultured tumor supernatants were below the level of detection of the ELISA (<100 pg/mL; ref. 46). However, these low levels were enough to inhibit the generation of tumor-associated macrophages and to restrict tumor angiogenesis (45, 46).

Type III IFNs (also known as IL-28 and IL-29) could also have a role in the inhibition of tumor growth observed in LPS-B16 tumors. They share the same intracellular signaling with type I IFNs but use a cell surface receptor, IL-28R, mainly expressed in epithelial layers (34, 43). B16 cells express IL-28R (48). LPS-B16 tumors in *Il28r* KO mice are not significantly smaller but still show a reduced growth compared with B16 tumors. Therefore,

we could hypothesize that IL-28 (produced by either LPS-activated tumor cells or host cells) could participate in this minor effect.

In our model, IFNβ produced by TLR4-activated tumor cells is involved in restoring the suppressive effect of B16 cell–derived factors on *Tlr4*$^{lps\text{-}del}$ BMDC maturation. Also, lack of inhibition of tumor growth is observed when LPS-stimulated B16 cells are inoculated in *Ifnar1*$^{-/-}$ mice. Thus, endogenous IFNβ, secreted by LPS-stimulated B16 cells could be enough to improve the maturation state of local DCs, promoting the secretion of IL-12 and then a more efficient antitumoral response (Fig. 6). Our findings show, for the first time, that tumor cells can be manipulated with classical adjuvants to contribute positively to the antitumoral immune response.

Another aspect that should be discussed is whether this positive contribution could actually happen in more realistic scenarios such as therapeutic settings in which the adjuvant is administered once tumors are visible. Only a transitory halt of tumor growth was observed in both, B16 and MB49 tumors in *Tlr4*$^{lps\text{-}del}$ animals which were intratumorally treated with LPS, indicating that type I IFN is necessary but not sufficient. In both cases, it has to be highlighted that even in the absence of TLR4 on innate immune cells (which are the expected target of LPS), tumor growth is controlled by the LPS treatment (at least transiently) in a context in which it can only be recognized by tumor cells.

The role of TLRs and, particularly, TLR4 in tumorigenesis and tumor promotion is highly controversial. Further research in this topic will open up new avenues for understanding tumor biology and for identifying potential new therapy strategies for cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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