IFNβ PRODUCED BY TLR4-ACTIVATED TUMOR CELLS IS INVOLVED IN IMPROVING THE ANTITUMORAL IMMUNE RESPONSE

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Abstract

Toll like receptor (TLRs) 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 (TLR4lps-del). Under such conditions, B16 cells yielded smaller tumors than non-stimulated 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 interferon β 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 (BMDCs) from TLR4-deficient mice, upregulating the expression of IL-12 and co-stimulatory molecules on those cells. BMDC maturation was blunted by addition of an IFN β-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.
Introduction

Toll-like receptors (TLRs) 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, 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 48h 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, since it was not observed in TLR4 deficient mice (TLR4<sup>−/−</sup>) (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 non-stimulated 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 interferon β, produced by TLR4-activated 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 deoxycytidylphosphate-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<sup>lps-del</sup>) were from the Jackson Laboratories. IL12p40<sup>−/−</sup> mice (26) were provided by Dr. Silvia Di Genaro (UNSL, Argentina). IFNRA1<sup>−/−</sup> and IL28R<sup>−/−</sup> 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 ATCC 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, USA.) and were used after five 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 48h, washed exhaustively and inoculated s.c. into TLR4<sup>lps-del</sup> mice. To generate conditioned medium (CM), cells were kept in 2.5% FBS and stimulated or not with 1μg/ml Ultrapure LPS (LPS-B16 CM or B16-CM respectively). After 48h, supernatants were collected.
**Generation of DCs.** DCs were obtained from bone marrow of TLR4\(^{lps-del}\) mice as described by Inaba et al. (27): >80% of harvested cells expressed CD11c. CD11c+ cells (2.5x10\(^5\)/ml) were incubated with LPS-B16 CM or with B16-CM and exposed to 10 µg/ml of CpG for 24h to analyze cytokine secretion by ELISA. LPS-B16 CM and B16-CM was incubated for 1h with an IFNβ-blocking antibody (4x10\(^2\)NU/ml, PBL Interferon Source), and then used to stimulate BMDCs.

**In vivo tumor challenge.** Melanomas were established in C57BL/6, TLR4\(^{lps-del}\), IL12p40\(^-/-\), IFNRA1\(^-/-\), and IL28R\(^-/-\) mice by s.c. of 1x10\(^6\) 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\(^{lps-del}\) mice were inoculated with 1x10\(^6\) B16 or 5x10\(^5\) MB49 cells and, once tumors reached approximately ~ 5mm\(^3\), they were treated intratumorally with LPS (1µg/200µl) or with 200µl of PBS, every other day for 6 consecutive times.

**Obtention of tumor-infiltrating cells.** Tumor infiltrating cells were obtained as previously described (24).

**qRT-PCR.** B16 cells (1x10\(^6\) cells) were stimulated or not with Ultrapure LPS for 6 and 24h. In the case of DCs, they were previously incubated with LPS-B16 CM or B16-CM for 20h prior to the addition of CpG for 4h. mRNA expression was analyzed with Mouse Toll-Like Receptor Signalling 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'-
GAGGATACCACCTCCCAACAGACC-3', Rev5'-AAGTGCATCATCGTTGTTCATACA-3'; GAPDH Fw5'-TCACCACCATGGAGAAGGC-3', Rev5'-GCTAAGCAGTTGGTGTTGCA-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.** mAbs conjugated with their respective fluorochromes were from BD-Bioscience and e-Biosciences. Intracellular cytokine was detected after stimulating cells 5h with PMA (10 ng/ml) and Ionomycin (1μg/ml, Sigma-Aldrich). BrefeldinA (10μg/ml; Sigma) was added for the last 4h of cell culture. Results were analyzed using FlowJo software (Tree Star, Inc.).

**Statistics.** Statistical analysis was performed using the Tukey post test to ANOVA analysis with the InfoStat software (National University of Córdoba). Values of p<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 non-stimulated or stimulated with 1μg/ml LPS for 6 and 24h. 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 observed for the NFkB mediator genes after 6h stimulation (Nfkb1, Nfkb2, Nfkbia, Nfkbib, RelA, MyD88 and others), most of the effector molecules were induced after 24h post-stimulation (150x IL6, 6x CCL2, 6x Csf2, 13x TNFα). 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 up-regulation of IFN beta expression levels was confirmed by qRT-PCR using a different set of primers (Fig.1B). The up-regulation of IFNb eta mRNA expression levels was also observed in two other murine tumor cell lines expressing TLR4: the MB49 and TRAMPC2 cells (Supp. Fig.1A). Both cell lines generated smaller tumors once activated via TLR4, prior to their in vivo inoculation in TLR4 lps-del and C57BL/6 mice. (Supp. Fig.1B and C). 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 (Supp. Fig.1C). Low levels of secreted IFNβ could also be reliably measured by ELISA in LPS-activated tumor cells (Fig. 1B and Supp. Fig.1A). IL6 and TNFα secreted levels increase with time in LPS-stimulated B16 cells supernatants, whereas the levels of
IL10 drop after just 12h of stimulation of B16 cells with LPS (Fig.1C). In contrast, the levels of Transforming Growth Factor β (TGFβ) are not modified.

**LPS-stimulated B16 cells supernatants can partially restore the suppressive effect of B16 cell-derived factors on TLR4<sup>lps-del</sup> BM-DC 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 48h with 1μg/ml Ultrapure LPS (LPS-B16 CM) or from non-stimulated cells (B16-CM) and incubated with BMDCs from TLR4<sup>lps-del</sup> mice for 20h before inducing their maturation with CpG. We used BMDCs from TLR4<sup>lps-del</sup> 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 4h of stimulation of TLR4<sup>lps-del</sup> BMDCs with CpG, transcription of genes coding for effector molecules such as *IL12a* (x8), *IL1a* (x12), *IL1b* (x7), *IL6* (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 like *NFkBα* (IκBalpha) showed a moderate raise (x11). When the same analysis was performed in TLR4<sup>lps-del</sup> 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 (like *IL1a* and *b*, *TNF* and *IL6*). Other genes were extremely down regulated in both experimental groups subjected to
tumor supernatants (like Csf2 and Csf3). In contrast, differences were found in the expression of certain genes among the three experimental groups. When transcription of genes of the NFkB complex was evaluated, only TLR4 \(^{lps-del}\) 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 to x33 and x375 in B16-CM). Interestingly, the expression of IL12a 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 IL12p40 secretion was observed when iDCs were simultaneously incubated with B16-CM and CpG for 24h. This reduction was not due to an expansion of Gr1+CD11c+ at the expense of CD11c+ cells, since the exposure of the iDCs for 24h to CM did not alter the final phenotype of the cells (Supp. Fig. 2A and B). In contrast, IL12p40 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 IL12p70 levels were evaluated: even though CpG alone is a poor inducer of IL12p70 (32), detectable levels of IL12p70 could be reliably measured in TLR4 \(^{lps-del}\) DCs exposed to CpG (Fig. 2B and Supp. Fig. 2C). These levels were almost abrogated when TLR4 \(^{lps-del}\) DCs were incubated with B16-CM, LPS-B16 CM and B16-CM plus CpG. In contrast, IL12p70 was partially restored when TLR4 \(^{lps-del}\) DCs were matured with CpG in the presence of LPS-B16 CM (Fig. 2B and Supp. Fig. 2C). When TNFα
(Fig. 2B) and IL6 (data not shown) were measured a similar effect was observed. The levels of these cytokines present in B16-CM or 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<sup>lps-del</sup> BMDCs in basal conditions. When TLR4<sup>lps-del</sup> BMDCs were incubated with B16-CM for 20h, TGFβ levels raised approximately 1.5 times compared to 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 20h, TGFβ levels dropped significantly (Fig. 2B). Surprisingly, maturation of TLR4<sup>lps-del</sup> BMDC in the presence of LPS-B16 CM significantly reduces the levels of TGFβ secreted, when compared to those secreted by DCs matured in the presence of B16-CM, supporting our hypothesis that DC pro-activating factors are increased in LPS-B16 CM.

In vitro stimulation of B16 cells with TLR4 agonists, like LPS or Monophosphoril Lipid A for 48h, before inoculation, produced significant inhibition of tumor growth in syngeneic C57BL/6 or in TLR4<sup>lps-del</sup> mice. Since 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 IL12. To test if 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<sup>lps-del</sup> and IL12p40 deficient mice. As expected, LPS-B16 tumors were significantly smaller than B16 tumors in TLR4<sup>lps-del</sup> mice; in contrast, both experimental groups (B16 and LPS-B16) followed the same pattern of tumor growth in IL12p40<sup>−/−</sup> mice, indicating that either IL12 or IL23 (which share the IL12p40 subunit) is required in the inhibition of tumor growth observed in <i>wt</i> 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 dendritic cells with a more mature phenotype as well as increased frequencies of IL12+ CD11c+ and IFNγ+ tumor infiltrating cells.** Then, TLR4<sup>lps-del</sup> mice were s.c. 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 Supp. Fig.3). 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 IL12+ CD11c+ cells (Fig.3D) was increased. Moreover, when tumor infiltrating mononuclear cells were stimulated ex-vivo with PMA-ionomycin for 5h and IFNγ-IL10 cytokine intracellular staining was performed, an increased frequency of IFNγ+ CD3+ cells was observed in LPS-B16 tumors compared to B16 tumors. The opposite was observed when IL10+ CD3+ cells were analyzed (Fig.3E and F).
Thus, LPS-B16 cells inoculated into TLR4\textsuperscript{lps-del} mice promote a better functionality of DCs \textit{in vivo}, which could be associated with a more efficient Th1 response \textit{in situ} and the reduced tumor growth observed.

**IFNβ produced by TLR4-activated tumor cells is involved in restoring the suppressive effect of B16 cell-derived factors on TLR4\textsuperscript{lps-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\textsuperscript{lps-del} BMDC maturation. We looked at CD40 expression in TLR4\textsuperscript{lps-del} BMDC incubated with LPS-B16 or B16-CMs for 20h and matured with CpG. A neutralizing anti-IFNβ was added to the CMs 1h before incubating them with DCs. As expected, the percentage of DCs expressing CD40 increases to an 80% after 4h 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\textsuperscript{lps-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 Supp. Fig. 4). Similar results were obtained when IL12p70 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 if IL-28 (a distinct category of type I–like IFN, also referred to as type III IFN) (34) could be playing a role in tumor inhibition in our model, we inoculated B16 cells stimulated or not with LPS in IL-28R KO mice. Inhibition of tumor growth was observed in wild type mice but only a partial and non-statistically significant inhibition of tumor growth was seen in the IL-28R 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 if TLR4 on tumor cells play a role in therapeutic settings, we performed local TLR4 stimulation by injecting LPS intratumorally in B16 and MB49 tumors in C57BL/6 and TLR4 lps-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-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 begun to grow (Fig.5B). However, although non-statistically different, tumor volumes kept being slightly smaller in LPS treated mice.
Discussion

B16-derived soluble factors strongly suppressed the activation of BMDC in response to CpG, down-regulating the secretion levels of IL12p40, IL12 and TNFα and diminishing the percentage of CD40+ CD11c+ cells. Interestingly, when BMDC from TLR4\textsuperscript{lps-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 show both an activated phenotype, it is improved in the later group. Also, an increase in IL12+ CD11c+ cells infiltrating tumors was observed, further providing evidence of their improved functional state \textit{in vivo}. Initial CD4+ T cell activation occurs in secondary lymph nodes, but in order to elicit effector functions, in lymph nodes or in non-lymphoid tissues like tumors, they need to see again the specific MHC-II-peptide complexes to produce effector cytokines, like 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 IL12. The fact that tumor infiltrating mononuclear cells produce higher levels of IFNγ and reduced levels of IL10, argues in favor of a more efficient immune response taking place in LPS-B16 tumor bearing animals, responsible of controlling tumor growth.

Thus, upon TLR4 activation B16 cells could secrete a different pattern of soluble factors and cytokines, which would favor the balance to a friendlier environment for activating DCs. Transcriptional analysis of LPS-stimulated B16 cells confirms this idea. The expression of \textit{GM-CSF} and \textit{G-CSF} genes considerably increases as well as those involved in the type I IFN pathway. Also, the levels of IL6 and TNFα are increased in LPS-
stimulated B16 cell supernatants. IL6 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 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 IL12p40 subunit, arguing that antigen presenting cells, main producers of IL12 and IL23 and presumably DCs, play a key role in our model. Both IL12p40 and IL12p70 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, since type I IFNs play a crucial role in the induction of IL12p70 (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 et al, have found that in vitro cultured tumor tissues, spontaneously released low concentrations of IFN-α (in the range of 5pg/ml) and that concentrations of IFN-β in cultured tumor supernatants were below the level of detection of the ELISA (<100pg/ml) (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 IL28 and IL29) 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, IL28R, mainly expressed in epithelial layers (34, 43). B16 cells express IL28R (48). LPS-B16 tumors in IL28R KO mice are not significantly smaller but still show a reduced growth comparing to B16 tumors. Therefore, we could hypothesize that IL28 (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<sub>lps-del</sub> BMDC maturation. Also, lack of inhibition of tumor growth is observed when LPS-stimulated B16 cells are inoculated in IFNAR<sup>-/-</sup> 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 IL12 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 discuss 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^{\text{hrs-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.
References


Legends for the Figures

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 to the expression in non-stimulated B16 cells.

B. *IFNb1* and *IL6* mRNA expression in B16 or LPS-B16 cells normalized to GAPDH 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 restore the suppressive effect of B16-CM on TLR4*_{lps-del}* BMDC maturation.

A. Heat map of qPCR array analysis. TLR4*_{lps-del}* BMDCs incubated with medium, B16-CM or LPS-B16 CM for 20h and then stimulated with CpG for 4h. Gene expression was compared to that from immature TLR4*_{lps-del}* BMDCs.

B. Cytokine levels in culture supernatants of TLR4*_{lps-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 three independent experiments. * p<0.05.

Figure 3. LPS-B16 tumor-bearing TLR4<sup>ips-del</sup> 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 three independent experiments. * p<0.05.

B. Costimulatory molecule-expression on spleen CD11c+ cells from naïve (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 naïve or tumor bearing mice. Results show mean ± SD of three independent experiments *p<0.05.

D. Intracellular IL12p40 staining on tumor infiltrating CD11c+ cells. Numbers indicate the percentage of IL12p40+ CD11c+ cells. Shaded histogram: isotype control, continuous line: IL12p40+CD11c+ cells (right panel).

E. Ex-vivo production of IFNγ and IL10 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 IL10 in culture supernatants of tumor infiltrating mononuclear cells activated ex-vivo with PMA-Ionomycin evaluated by ELISA (left). *p<0.05. Percentages of IFNγ,
IL10 in CD3+ cells and IL12p70 in CD11c+ cells in B16 or LPS-B16 tumors (right). Results show mean ± SD of three independent experiments.

**Figure 4. IFNβ derived from LPS-stimulated B16 cells induce TLR4\textit{lps-del}\ dendritic cells 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. IL12p70 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 two independent experiments. *p<0.05 for IFNAR\textit{+/+} LPS-B16 vs IFNAR\textit{−/−} LPS-B16, IFNAR\textit{+/+} B16 and IFNAR\textit{−/−} B16 (mean±SEM).

D. Tumors were induced with B16 or LPS-B16 in IL28R−/− mice (n=10). * p<0.05 for IL28R\textit{+/+} LPS-B16 vs IL28R\textit{+/+} B16 and IL28R\textit{−/−} B16 (mean±SEM).

**Figure 5. Local stimulation of TLR4 present on tumor cells retards temporally tumor growth in TLR4\textit{lps-del} mice.**

LPS or PBS was injected in C57BL/6 (A) and TLR4\textit{lps-del} (B) tumor bearing mice for 6 days (arrows) at the tumor site (n=8). * p<0.05

**Figure 6. Proposed model.** (1) LPS-B16 cells secrete IFNβ and other modulators of DC activity; (2) IFNβ can significantly restore the IL12-secreting capacity of DCs at the site of inoculation, improving their maturation state; (3) DCs promote a Th1 response against the growing tumor (4).
Figure 1.

A.

B.

C.

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IL6

TNFα

IL10

TGFβ

IFNβ

Fold Induction

IF-ND

IL-β

Mean ± SEM; *P < 0.05.
Figure 2.

A.

B.

C.

TLR4^{lo-s-dei}  

IL12p40^{+}  

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Figure 3

A. Spleen and Tumor

B. MHC II, CD40, CD80, CD86

C. MFI of MHC-II, CD40, CD80, CD86

D. LPS-B16

E. Total tumor infiltrating mononuclear cells, CD3, CD11c, NK1.1

F. IFN-γ, IL10
Figure 4:

A. Blocking Anti IFNβ

-isotype control
-DCs + media
-Sample

B. CD40%

MEDIUM
-CD40

C. B16.CM + CpG

LPS-B16.CM + CpG

D. IL12 p70

PG/mL

Medium
Medium + anti IFN

CpG

B16.CM + CpG

LPS-B16.CM + CpG

IFN

E. IFNAR +/- B16

IFNAR +/- LPS-B16

F. IL28R +/- B16

IL28R +/- LPS-B16
Figure 5

A.

B16

MB49

PBS/LPS Treatment

PBS

LPS

Tumor volume (mm³)

Days post injection

B.

B16

MB49

PBS/LPS Treatment

PBS

LPS

Tumor volume (mm³)

Days post injection

Research.
Figure 6
IFN β produced by TLR4-activated tumor cells is involved in improving the antitumoral immune response

Nicolas Gonzalo Nuñez, Virginia Andreani, Maria Ines Crespo, et al.

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