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Intratumoral Immunization by p19Arf and Interferon-β Gene Transfer in a Heterotopic Mouse Model of Lung Carcinoma[1,2]

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Abstract

Therapeutic strategies that act by eliciting and enhancing antitumor immunity have been clinically validated as an effective treatment modality but may benefit from the induction of both cell death and immune activation as primary stimuli. Using our AdRGD-PG adenovector platform, we show here for the first time that *in situ* gene transfer of p19Arf and interferon-β (IFNβ) in the LLC1 mouse model of lung carcinoma acts as an immunotherapy. Although p19Arf is sufficient to induce cell death, only its pairing with IFNβ significantly induced markers of immunogenic cell death. *In situ* gene therapy with IFNβ, either alone or in combination with p19Arf, could retard tumor progression, but only the combined treatment was associated with a protective immune response. Specifically in the case of combined intratumoral gene transfer, we identified 167 differentially expressed genes when using microarray to evaluate tumors that were treated *in vivo* and confirmed the activation of CCL3, CXCL3, IL1α, IL1β, CD274, and OSM, involved in immune response and chemotaxis. Histologic evaluation revealed significant tumor infiltration by neutrophils, whereas functional depletion of granulocytes ablated the antitumor effect of our approach. The association of *in situ* gene therapy with cisplatin resulted in synergistic elimination of tumor progression. In all, *in situ* gene transfer with p19Arf

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and IFNβ acts as an immunotherapy involving recruitment of neutrophils, a desirable but previously untested outcome, and this approach may be allied with chemotherapy, thus providing significant antitumor activity and warranting further development for the treatment of lung carcinoma.

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Introduction

Despite extraordinary advances in controlling cancer progression, long-term benefit remains disappointing [1–4]. Even so, current treatments do extend survival and create new therapeutic opportunities, especially for strategies that induce a long-lasting response, such as the modulation of antitumor immunity [5]. Several findings support the hypothesis that local immunomodulation is able to control and even eradicate primary and distant tumors [6–9]. In addition, studies have shown that effective T-cell activation can be achieved intratumorally, dispensing the participation of draining lymphatic organs [10,11]. In this regard, we investigated whether intratumoral gene transfer of p19Arf and interferon-β (IFNβ) would generate immunogenic cell death (ICD) in a strong immunostimulatory context, allowing the activation of a cellular antitumor response.

The p19Arf (alternative reading frame) tumor suppressor protein is well known as an inhibitor of the Mdm2-mediated ubiquitination of p53, thus contributing to the activation of p53 in response to cellular stress [12,13]. Considering the pivotal role of p53 signaling in cancer prevention, mutations in this pathway are a very common event in cellular transformation. Deregulation of the p53 pathway is also associated with increased resistance to chemo- and radiotherapy [14,15]. In this context, restoration of the p53 pathway has been extensively explored in preclinical and clinical settings [16–18]. P19Arf gene transfer has been shown to inhibit cell growth and induce apoptosis in several models [19–23]. Furthermore, p19Arf has been implicated in antitumor functions independently of p53 activation [17].

The multifunctional cytokine IFNβ has been implicated in the stimulation of a plethora of genes which impact virtually the entire cellular organization [24]. In cancer research and therapy, type I IFNs, specifically α and β, are extensively used due to their tumor suppressor capabilities by acting directly on tumor cells and through immunomodulatory properties. Type I IFNs induce apoptosis and cell cycle arrest in several models [25–27]. Interestingly, IFNβ can promote p53 transcription, enhancing cell death in response to DNA-damaging agents [28]. In addition to the direct effects of type I IFNs on cancer cell viability, an impressive number of studies have shown the ability of type I IFN to modulate the antitumor immune response. Type I IFNs are implicated in recruitment, proliferation, differentiation, and activation of immune cells [29–32]. Curiously, recent evidence from mouse models has shown that IFNβ signaling plays a pivotal role in the antitumor response induced by radiotherapy [33,34] and by chemotherapy with anthracyclines [35]. IFNβ gene transfer has been exploited in a considerable number of studies, indicating that IFNβ is sufficient to modulate the tumor microenvironment, inducing or improving immunological response [36–40].

We have previously shown that intratumoral gene transfer of p19Arf and IFNβ or IFNβ alone is able to reduce tumor growth in a murine model of melanoma. Although IFNβ gene transfer alone could produce this effect, only its association with p19Arf induced increased cell death *in vivo* [23]. In vaccination strategies, we also showed that B16 transduced with the combination of p19Arf and IFNβ prolonged survival in mice [41]. Until now, we had no evidence that our gene transfer approach, when applied directly to the tumor mass, would induce a protective immune response. Here we demonstrate for the first time that combined *in situ* gene therapy is able to circumvent tumor suppressive microenvironment and provide a significant advantage for immunostimulation. This advantage is characterized by differential expression of genes that affect the immune response and chemotaxis, validated through the observation of recruitment of neutrophils and antitumor activity of granulocytes. The combined gene transfer approach also yielded a synergistic impact on tumor growth when associated with cisplatin.

Materials and Methods

Mice and Cell Line

Wild-type, female C57BL/6 mice were purchased from Centro de Bioterismo, Faculdade de Medicina, Universidade de São Paulo (FM-USP). Animals were 6 to 9 weeks of age at the time of experimental procedures. All experiments were approved by the Ethics Committee of FM-USP (research protocol 116/10).

Lewis lung carcinoma cell line (LLC1) was kindly provided by Dr. Karim Benihoud and cultured in Dulbecco's modified Eagle medium (Gibco cat. no. 12,100–046) supplemented with 10% of fetal bovine serum and Gibco Antibiotic-Antimycotic solution.

Adenovector Production

The serotype 5 adenovectors used in this work contain the RGD modification in the fiber protein [42]. The backbone provided by Dr. Hiroyuki Mizuguchi (Osaka University, Japan) was modified to allow *in vitro* recombination using clonase (Invitrogen, 12538120) (A.H., manuscript submitted). Adenovectors express eGFP, IFNβ, and p19Arf under the control of a p53 responsive promoter [43] or LacZ the under control of the CMV promoter.

Adenovector production was performed by transfection of linearized plasmids into HEK293A cells followed by amplification cycles and purification using iodixanol gradient [44]. Purified adenovectors were stored in PBS 7% glycerol at −80°C. Titration was done using Adeno-X Rapid Titer Kit (Clontech, 632250).

In Vitro Experiments

LLC1 cells were transduced with adenovector concentration of 1.5×10^5 ifu/μl in DMEM. After 24 hours, transduction was supplemented with DMEM 10% FBS.

Phosphatydylserine exposure was determined by flow cytometry after staining with Annexin V, Alexa Fluor 488 conjugate (Thermo Scientific, A13201), and propidium iodide (Sigma, cat. no. P4170). DNA content was determined by propidium iodide staining after cell permeabilization with 70% ethanol.

The clonogenic assay was performed by plating 2000 transduced cells in 10-cm dishes, and colony formation was quantified 12 days later.

Calreticulin exposure was determined by surface staining using rabbit anticalreticulin polyclonal antibody 1:200 (Novus Biologicals, NB300-545) and goat antirabbit conjugate Alexa Fluor 488, 1:500 (Thermo Scientific, A11008) costained with propidium iodide.

Vesicular ATP content [45,46] was assessed by incubating cells with 5 μM quinacrine (Sigma, Q3252-25G) in Krebs-Ringer solution during 30 minutes and then costaining with propidium iodide.

In Vivo Assays

All procedures were evaluated and approved by the Research Ethics Committee as well as the Committee on the Ethical use of Animals, University of Sao Paulo, School of Medicine. Mice were inoculated subcutaneously with 1×10^6 LLC1 tumor cells. When tumors reached a diameter of 3 to 5 mm, adenovector treatment was started. Intratumoral injections were performed every 48 hours for a total of 6 applications. Mice were challenged with an inoculation of 5×10^5 LLC1 cells in the opposite flank of the treated tumor 10 days after the first adenovector application. Cisplatin was intratumorally applied (50 μl at 1 mg ml^{-1}) 24 hours after the fifth adenovector application.

For prophylactic vaccination, 48 hours prior to subcutaneous inoculation, 2×10^6 cells were transduced with adenovectors, and cisplatin (2.5 μM final concentration) was added to transduced cells (12 hours prior subcutaneous inoculations). Mice were challenged with 5×10^5 cells in the opposite flank 7 days after vaccination.

Granulocyte depletion was done by two intraperitoneal applications of GL6-8AC antibody, separated by 4 days, starting with the first adenovector application. Depletion was confirmed by differential blood counts at 24, 48, and 72 hours after IgG injection (data not shown).

Microarray

Tumors were excised 48 hours after the fifth adenovector application; RNA extraction was performed using TRIzol reagent (Thermo Scientific, 15596026) following the manufacturer's instructions. RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, G2939AA). Gene Chip Mouse 1.0 ST (Affimetrix Inc., 901171) was used according to the manufacturer's instructions to determine gene expression. Microarray results were analyzed using TM4 Microarray software suite (Dana-Farber Cancer Institute, USA). Differential gene expression profile was obtained by comparing the p19, IFNβ, and p19 + IFNβ to eGFP-treated tumors using rank product method (false discovery rate < 0.05). Enrichment analysis was performed in DAVID database (EASE score 0.001). Webs of gene interactions were constructed using String database (confidence 0.150).

Quantitative Polymerase Chain Reaction (PCR)

Primers were designed using PrimerBlast (NCBI, USA) and are shown in the 5′ to 3′ orientation: IL1a, Fwd GTCAACTCATTGGCGC TTGA, Rev. GAGAGAGATGGTCAATGGCAGA; Osm, Fwd GCAGAATCAGGCGAACCTCA, Rev. GCTCTCAGGTCAGGTG TGTT; Cd8a, Fwd TTCTGTCGTGCCAGTCCTTC, Rev. GGCCG ACAATCTTCTGGTC; GZMA, Fwd GGGGGCCATCTCTT GCTAC, Rev. AACAACCGTGTCTCCTCCAA; Cd274, Fwd CTC ATGCCAGGCTGCACTT, Rev. ACAAGTCCTTTGGAGCCGTG, Ifng, Fwd CAGCAACAGCAAGGCGAAA, Rev. GTGGACCACTC GGATGAGC; Ccl12, Fwd CACTTCTATGCCTCCTGCTCAT, Rev. CCGGACGTGAATCTTCTGCTT; Ccl17, Fwd CTCTGC TTCTGGGGACTTTTCT, Rev. CAGCACTCTCGGCCTACAT; Cxcl3, Fwd CCACTGCACCCAGACAGAAG, Rev. GGTGAGGGG CTTCCTCCTT; Il1B, Fwd AGTTGACGGACCCCAAAAGA, Rev. GATGTGCTGCTGCGAGATTT; Ccl3, Fwd GCAACCAAGTCTT CTCAGCG, Rev. TGGAATCTTCCGGCTGTAGG). Quantitative PCR was done using Power Sybr Green PCR master mix (ThermoFisher Scientific, 4367659). Expression was calculated by $2^{-\Delta\Delta Ct}$ method, and reference gene was the average expression of β-actin and GAPDH.

Immunohistochemistry

Tumors were excised 72 hours after the fifth adenovector injection, fixed for 4 hours in 4% buffered formamide at room temperature, and then incubated overnight in 30% sucrose at 4°C. Tumors were frozen in Tissue-Tec OCT and stored at −80°C. Slides were prepared by sectioning tumors in a cryostat at 5 μm. Primary antibodies were incubated during 1 hour in PBS 10% FBS solution [Anti CD11b-Alexa 647 Ebioscience, clone M1/70 (1:100); Anti CD11c MBL International, clone 223H7 (1:50); Anti CD86-Alexa 647 Biolegend, clone GL-1 (1:100); Anti CD169 Abcam, 3D6.112 (1:300); Anti F4/80-Alexa 647 Biolegend, clone CI:A3-1 (1:300); Anti cleaved caspase 3 Cell Signaling, clone Asp175 (1:300); Anti Ly6G-PE Biolegend, clone 1A8 (1:100)].

Statistics

Results are expressed as means ± SEM. Statistical significance was assessed by analysis of variance (ANOVA) followed by Tukey posttest or Mann-Whitney *U* test. Distributions in tumor growth were compared by ANOVA followed by Bonferroni posttest. Analyses were performed using GraphPad Prism 6.0.

Results

p19Arf Induces Death in LLC1 Cells

To examine the impact of gene transfer on cell viability, LLC1 cells were transduced with 10^5 ifu/μl of AdRGD-PG-LacZ, AdRGD-PG-p19Arf, AdRGD-PG-IFNβ, or AdRGD-PG-p19Arf and AdRGD-PG-IFNβ. The measurement of phosphatidylserine exposure 72 hours after transduction shows that p19Arf is mainly responsible for induction of cell death (Figure 1*A*); however, association with IFNβ was able to increase this effect. The chemical inhibition of p53-Mdm2 interaction by Nutlin-3 also was able to induce cell death (Figure 1*B*), suggesting a p53-dependent process. A clonogenic assay revealed that combination of p19Arf and IFNβ is able to induce a significant decrease in cell viability even when compared with p19Arf alone (Figure 1*C*). As shown here, whereas p19Arf induces cell death, its association with IFNβ can enhance this effect.

Release of Immunogenic Signals in Response to Combined p19Arf and IFNβ Gene Transfer

Immunogenic cell death, such as induced by anthracyclines, has been shown to be a critical factor for the optimal success of treatment in mouse models. Here we characterized the release of classical ICD signals in response to our gene transfer strategy. As shown in Figure 2*A*, p19Arf gene transfer was able to induce the surface exposure of calreticulin at *premortem* stage (CRT+PI−, 48 hours posttransduction). Moreover, the combination of p19Arf plus IFNβ gene transfer significantly increased CRT+PI− cells when compared with p19Arf gene transfer alone. We also determined that p19Arf is mainly responsible for lysosome loss, revealed by a decrease in quinacrine-labeled cells, indicating ATP release (Figure 2*B*). Strikingly, the combined, but not individual, p19Arf and IFNβ gene transfer resulted in the release of HMGB1 (Figure 2*C*). Taken together, these results indicate that only the combined gene transfer approach promotes the release of all three markers of ICD.

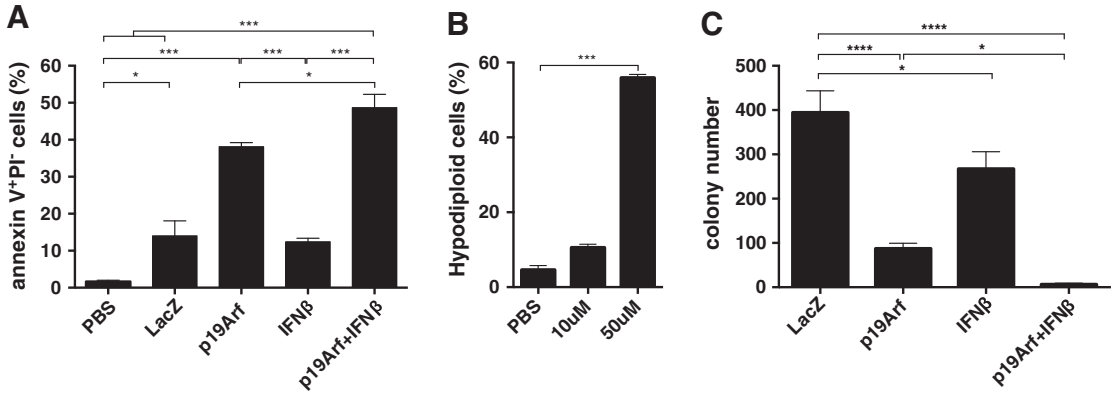

Figure 1. Cell death and viability in LLC1 cells. (A) Cells were transduced with 1.5×10^5 ifu/μl of each adenovector. Annexin V–positive cells were determined by flow cytometry. (B) LLC1 cells were treated with Nutlin-3; hypodiploid cells were determined by flow cytometry after propidium iodide staining. (C) Cell viability was determined in a clonogenic assay. *$P < .05$, ***$P < .001$, ****$P < .0001$ (ANOVA followed by Tukey comparison test in A and B and Mann-Whitney *U* test in C).

Intratumoral Gene Transfer of IFNβ or Its Combination with p19Arf Significantly Affects Tumor Progression in Immune Competent Host

LLC1 cells were implanted subcutaneously in C57BL/6 or athymic mice; when the tumors attained a diameter of 3 to 5 mm, intratumoral injections of recombinant adenoviruses were performed. The injections were repeated 3, 5, 7, and 9 days after the first application. Treatment with IFNβ, p19Arf, or the combination IFNβ plus p19Arf was able to reduce tumor growth when compared with mice treated with eGFP (Figure 3*A*). However, mice treated with IFNβ showed increased ability to reduce tumor growth when compared with p19Arf alone. Furthermore, intratumoral treatment of athymic mice abrogated the antitumor response when treated with IFNβ or the combination IFNβ plus p19Arf, indicating dependence on the T-cell compartment for controlling tumor growth (Figure 3*B*).

In Situ Gene Therapy with the Combination of p19Arf and IFNβ Induces a Protective Immune Response against Challenge Tumors

To determine if the association of p19Arf and IFNβ gene transfer is able to induce an immune response, the C57BL/6 mice described above were challenged with the implantation of naive LLC1 cells in the flank opposite to the site of the primary, treated tumor. Mice that had the primary tumor treated with the combination p19Arf plus IFNβ showed a significant reduction of tumor growth at the challenge site as compared with animals treated with IFNβ alone (Figure 4*A*). Groups treated with PBS or p19Arf alone were sacrificed early due to primary tumor burden. To confirm this result in a different model, we performed prophylactic vaccination where cells were first transduced *ex vivo* with p19Arf, IFNβ, or both p19Arf and IFNβ. To guarantee that all cells are injected in a similar condition (the same percentage of dead cells), they were also treated with cisplatin, a nonimmunogenic drug [47], 24 hours before vaccination. These cells were injected subcutaneously, and 7 days after vaccinations, mice were submitted to challenge with naive LLC1 cells in the opposite flank. The tendency of mice vaccinated with cells transduced with both p19Arf and IFNβ confirmed our previous observation, indicating an increased immunoprotection (Figure 4*B*) and highlighting a potential benefit of using our combined gene transfer approach.

Differential Gene Expression in Tumors Treated With the Combination of p19Arf and IFNβ

To identify the putative mechanism by which the p19Arf and IFNβ combination impacted immune stimulation, we performed microarray analysis of transcripts expressed *in vivo*. Animals submitted to intratumoral gene transfer were sacrificed 48 hours after the last adenovector injection (day 13), tumors were excised and microarray was performed. Analysis of

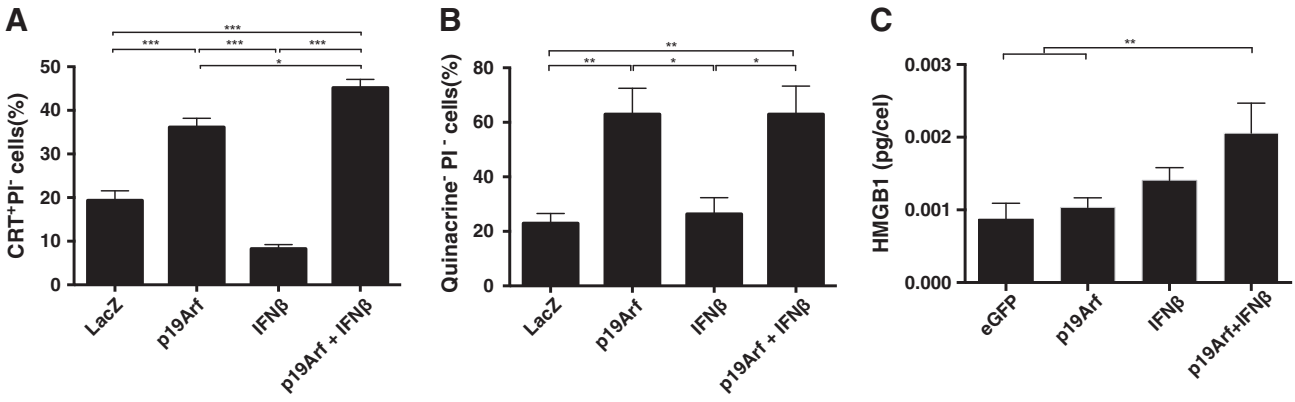

Figure 2. Immunogenic features of p19Arf-induced cell death. (A) Surface calreticulin determined by flow cytometry in live cells 48 hours after adenovector transduction. (B) ATP release was characterized by quinacrine staining 72 hours after gene transfer. (C) HMGB1 release was revealed by ELISA 48 hours posttransduction. *$P < .05$, **$P < .01$, ***$P < .001$ (ANOVA followed by Tukey comparison test, means ± SEM.)

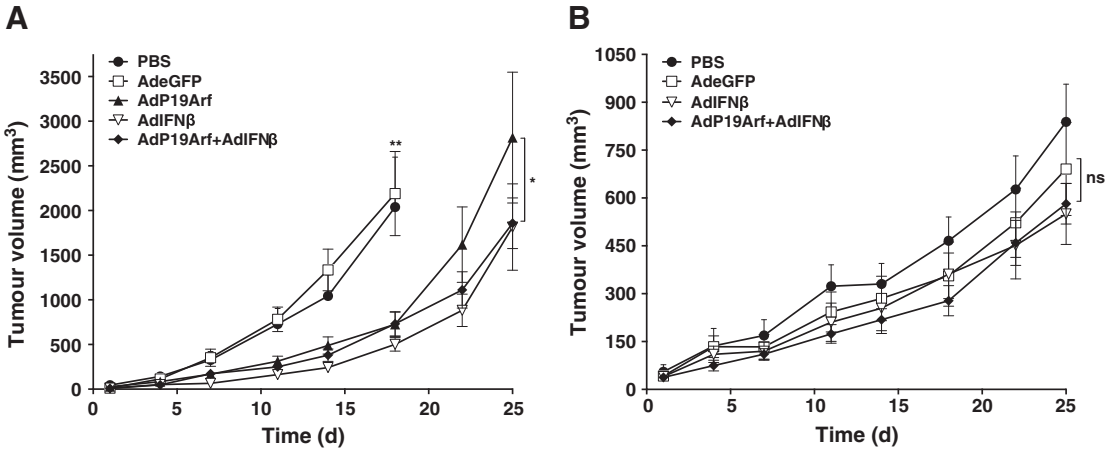

Figure 3. Effect of intratumoral gene transfer on tumor growth. (A) C57BL/6 or (B) athymic mice bearing LLC1 subcutaneous tumors were submitted to intratumoral gene transfer with 10^8 ifu on days 1, 3, 5, 7, 9, and 11. $^{**}P < .01$ compared with eGFP treated mice. $^{*}P < .05$ comparing IFNβ to P19Arf (ANOVA followed by Bonferroni posttest). In A, PBS $n = 6$, eGFP $n = 7$, P19Arf $n = 11$, IFNβ $n = 7$, and P19Arf + IFNβ $n = 7$. In B, $n = 5$ for all groups. Quantitative data are reported as mean ± SEM.

data indicates that combined gene transfer was exclusively responsible for the up- or downregulation of 75 or 87 genes, respectively (Supplemental Figure 1*A*). This set of genes was evaluated *in silico* to verify biological processes that are enriched. Clearly, the differentially expressed genes indicate a strong immune response and chemotaxic enrichment ($P < E^{-6}$) (Supplemental Figure 1*B* and Supplemental Table 1), suggesting involvement of neutrophils and T cells. To identify a subset of genes with pivotal importance in these processes, we constructed a web of genes (Supplemental Figure 2), which allowed us to determine the central proteins. Associating our data with information from the literature, we selected 12 genes for validation by qPCR (CCL2, CCL3, CCL17, IL1β, IL1α, CXCL2, CXCL3, CD8a, GZMA,

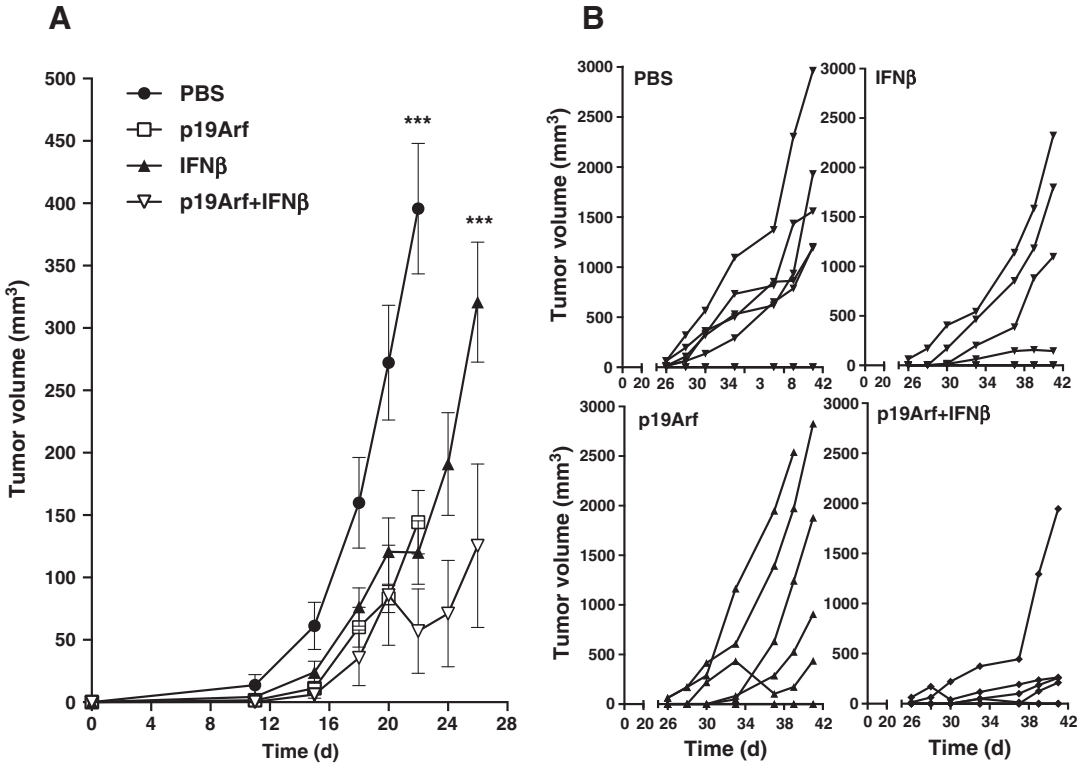

Figure 4. Combination of p19Arf and IFNβ gene transfer increases immunoprotection. (A) Mice whose primary LLC1 tumors were treated with *in situ* gene transfer were submitted to a challenge in the opposite flank; curves represent tumor growth at the challenge site. PBS $n = 5$, eGFP $n = 7$, p19Arf $n = 11$, IFNβ $n = 7$, and p19Arf + IFNβ $n = 7$ (means ± SEM). (B) LLC1 cells were transduced *ex vivo* and used to vaccinate animals, and 7 days after, naive tumor cells were implanted in the opposite flank and their growth was measured ($n = 7$ for all groups, $P < .05$ in PBS versus p19Arf + IFNβ). $^{*}P < .05$, $^{**}P < .01$ compared with PBS (ANOVA followed by Bonferroni posttest).

CD274, OSM, and IFNγ). The quantitative PCR did not confirm the differential expression of CCL2, CCL17, CXCL2, CD8a, GZMA, and IFNγ (data not shown). However, CCL3, CXCL3, IL1α, IL1β, CD274, and OSM showed increase expression only in animals treated with the gene transfer combination (Figure 5).

Impact of p19Arf and IFNβ Gene Transfer on Tumor Infiltrating Myeloid Cells

It is well known that efficient generation of an antitumor immune response is achieved by successfully accomplishing several sequential steps, including antigen presentation, T-cell differentiation/activation, and finally the ability of effector cells to avoid negative regulatory checkpoints [48]. Thus, to determine if the immunostimulatory genes identified in the microarray are reflecting a change in the tumor microenvironment, we investigated if the intratumoral gene transfer impacted infiltration of myeloid cells. Dendritic cells, neutrophils, and macrophages have been described by their ability to present antigens. The quantification of intratumoral $CD11c^+$ $CD86^+$ dendritic cells by immunohistochemistry showed an increase only in IFNβ-treated tumors (Figure 6*A*). Moreover, the $F4/80^+$ $CD169^+$ cells were significantly decreased in the groups treated with IFNβ (Figure 6*B*); curiously, this decrease in the macrophage population seemed to be attributed only to the $CD169^+$ subpopulation because the overall $F4/80^+$ population remained invariable (Supplemental Figure 3*A*). Although the overall myeloid population represented by $CD11b^+$ cells remained invariable (Supplemental Figure 3*B*), the immunohistochemistry revealed an increased number of $CD11b^+$ $Ly6G^+$ cells exclusively in animals treated with the combination of p19Arf and IFNβ (Figure 6*C* and Supplemental Figure S4*A*).

Granulocytic Cell Population Plays a Central Role in Controlling Tumor Growth in Animals Treated with p19Arf and IFNβ

We next determined if the differential infiltration of neutrophils, induced by combined gene transfer, was impacting tumor growth. Animals were treated with intratumoral applications of eGFP or the combination p19Arf plus IFNβ as described previously. The granulocytic cell population in these animals was depleted by intraperitoneal injections of RB6-8C5 antibody. Granulocyte depletion was verified by complete blood count (data not shown). Tumor growth shows that granulocytic population is responsible for the antitumor effects induced by gene transfer (Figure 7). This result does not exclude the involvement of other immune cells, especially T lymphocytes, as indicated by abrogation of antitumor effects in nude mice (Figure 3*B*).

Synergistic Effect of Intratumoral Gene Transfer with Cisplatin Treatment

To approximate common clinical procedures, we associated the gene transfer with a current chemotherapeutic agent, cisplatin (CDDP).

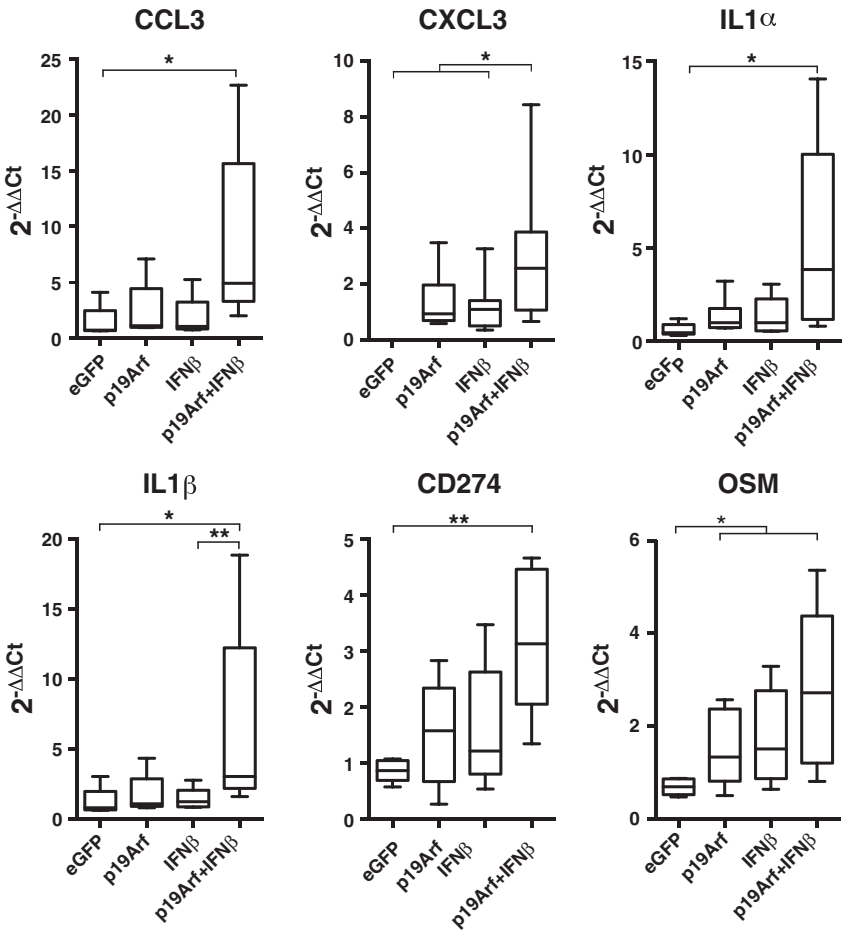

Figure 5. Differential gene expression induced by combined p19Arf and IFNβ gene transfer. Animals were treated as per Figure 3, and tumors were collected 24 hours after the last adenovirus application. CCL3, CXCL3, IL1α, IL1β, CD274, and OSM mRNA expression was confirmed by real-time PCR. *$P < .05$, **$P < .01$ compared with AdeGFP-treated tumors (Mann-Whitney test, means ± SEM).

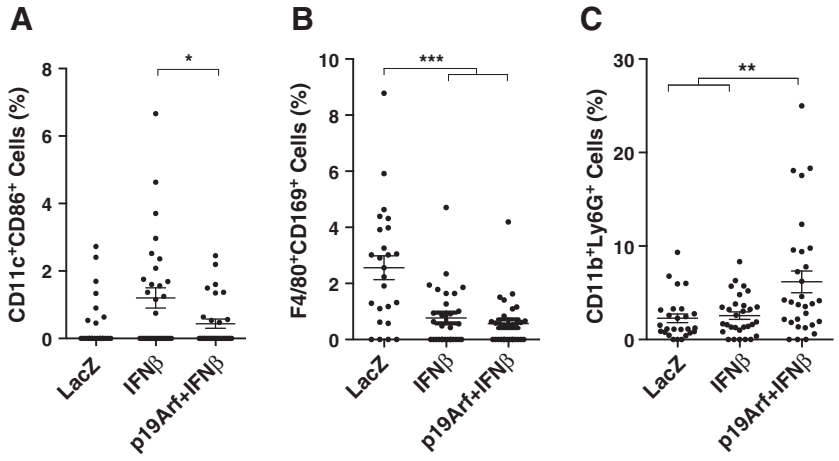

Figure 6. Intratumoral gene transfer induces accumulation of Ly6G+ cells in tumors treated by P19Arf plus IFNβ. Mice were treated with AdLacZ, IFNβ, or p19Arf + IFNβ, and 48 hours after the last (fifth) adenovector injection, tumors were harvested and processed for protein detection by immunohistochemistry to reveal subpopulations of (A) dendritic cells, (B) macrophages, or (C) neutrophils. *$P < .05$, **$P < .01$, ***$P < .001$ (ANOVA followed by Tukey comparison test, means ± SEM).

Mice treated with combination gene transfer associated with CDDP showed a decrease in tumor growth compared with either gene transfer or CDDP alone, indicating a strong synergistic effect (Figure 8*A*). Tumors that received CDDP and gene transfer showed an increased number of cells positive for cleaved caspase-3 and an increased number of neutrophils in the tumor bed (Figure 8, *B* and *C* and Supplemental Figure S4, *A* and *B*).

Discussion

In situ vaccination strategies constitute an approach in which an immune response is generated *in vivo* without the previous identification and isolation of a tumor-associated antigen [49]. For decades, *in situ* therapy has been successfully applied to treat bladder cancer, and its mechanisms rely on the modulation of urothelial environment by attracting and activating immune cells to a Th1 response [50]. Currently, several models of intratumoral vaccination, including the application of engineered viruses, have been shown to elicit an effective immune response in different solid tumors [49]. Indeed, with the advent of immunotherapies, new strategies exploiting tumor-associated antigens to generate an antitumor response could constitute a future multimodality therapy [51].

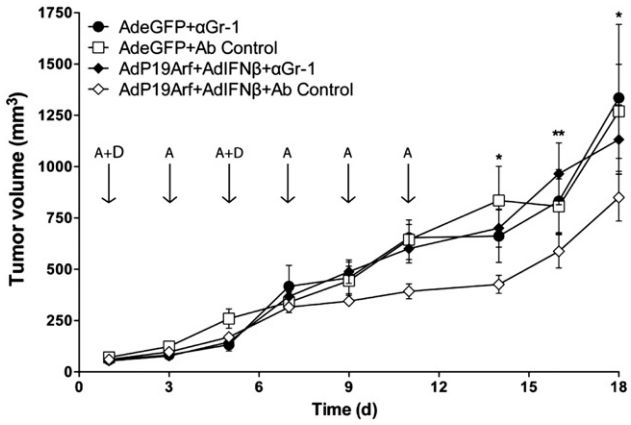

Figure 7. Therapeutic efficacy of p19Arf plus IFNβ gene transfer relies on the granulocytic population. Mice bearing LLC1 tumors were treated by intratumoral applications of eGFP or p19Arf plus IFNβ, combined with either Gr-1–specific or control antibodies. Arrows represent intratumoral application of adenovector ("A") and depeltion ("D") by intraperitoneal antibody injection. *$P < .05$, **$P < .01$ (ANOVA followed by Bonferroni comparison test). Data are reported as mean ± SEM.

Aiming to induce both cell death and antitumor immunity, we have previously generated a p53-responsive adenoviral vector, serotype 5, called AdPG; used this for the transfer of p19Arf or IFNβ to the B16 melanoma cell line; and observed that upon combined transduction, but not individual, a superior induction of cell death was achieved both *in vitro* and *in vivo* [23]. More recently, we employed *ex vivo* transduced melanoma cells in a tumor vaccine model. Although IFNβ alone or in combination with p19Arf resulted in antitumor protection mediated by TH1 CD4+ and CD8+ T cells, only the combination prolonged overall survival, augmented NK cell activity, and reduced tumor progression in a therapeutic vaccine model [41]. Here, we address for the first time the immunogenic properties upon *in situ* gene transfer of p19Arf and IFNβ in a mouse lung carcinoma model. We show that multiple gene transfer (p19Arf and IFNβ) was able to improve the emission of immunogenic signals and modulate myeloid infiltration, and may be a suitable option to increase immunogenicity in the tumor bed.

Our data reveals that inhibition of Mdm2 by either p19Arf or Nutlin-3 yielded similar results, suggesting that p53 is expected to be involved in the cell death mechanism. In addition, by using a p53 responsive promoter, we confirm that endogenous p53 maintains its transcriptional function in this cell line (data not shown). The gene transfer of both p19Arf and IFNβ was able to increase cell death and decrease viability of LLC1 cells. In fact, a few groups have described interaction of p53 and type I interferon pathways [28,52–54], providing additional opportunities for interplay between endogenous p53 and IFNβ for the induction of cell death and immune activation.

ICD has been described as a process in which a stimulus induces the spatiotemporal emission of signals that act by initiating an effective immune response to cellular antigens [55]. Classically, ICD

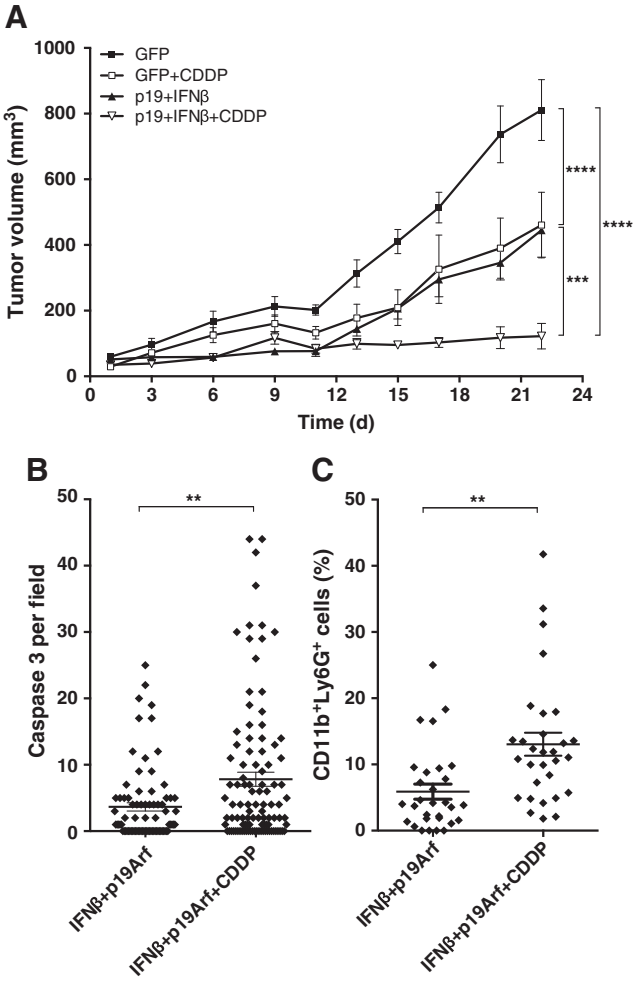

Figure 8. Intratumoral gene transfer of p19Arf plus IFNβ synergizes with current therapies. (A) Mice bearing LLC1 tumors were submitted to intratumoral gene transfer and treated with a single intratumoral application of cisplatin (CDDP) or PBS 48 hours after the last adenovector injection ***$P < .001$, ****$P < .0001$, $n = 5$ per group, (ANOVA followed by Bonferroni comparison test). (B) Quantification of cleaved caspase-3 or (C) accumulation of $Cd11b^+Ly6G^+$ as revealed by immunohistochemistry (**$P < .01$, Mann-Whitney).

induced by certain chemotherapeutic agents, such as anthracyclines, relies on the exposure of calreticulin, release of ATP, HMGB1, and type I IFN [47]. Here, we show that cell death induced by p19Arf is able to induce calreticulin exposure and ATP release; however, only in association with IFNβ was HMGB1 release significantly increased in comparison to controls. In line with these results, *in vivo* experiments demonstrated that association of IFNβ with p19Arf was able to improve the immune response when either *in situ* gene therapy or a prophylactic vaccine was used. Furthermore, IFNβ signaling has recently been shown to be required for the induction of an immune response following treatment with anthracyclines [56], constituting a fourth hallmark of ICD.

Differential gene expression induced by combined gene transfer revealed a cluster of proteins related to the induction of an immune response. Quantitative PCR confirmed the upregulation of Ccl3, Cxcl3, IL1β, IL1α, and OSM, cytokines that are all involved in neutrophil recruitment [57–61]. The individual contribution of each cytokine in our model remains to be elucidated. Because these molecules do not seem to be upregulated LLC1 cells transduced *in vitro* (data not shown), we hypothesize that *in vivo* tumor cell death is responsible for orchestrating neutrophil recruitment. Interestingly and in agreement with our observations, HMGB1 has been associated with recruitment [62] and severity [63,64] of tissue injury by neutrophils during inflammation.

Furthermore, we confirmed by immunohistochemistry an increased population of tumor-associated neutrophils (TANs) in the tumor bed, and this population was shown to be crucial to the antitumor effect induced by p19Arf and IFNβ, as shown by depletion of neutrophils. The contribution of TANs to tumor progression is controversial and seems to be a context-dependent phenomenon. Despite several findings indicating a poor prognostic associated to TANs [65], recent studies have shown that neutrophils exhibit plasticity and can be polarized to an antitumor phenotype [66]. Besides the production of tumoricidal molecules and induction of apoptosis in tumor cells, neutrophils are involved in tumor rejection and immune memory through interactions with $CD8^+$ and $CD4^+$ lymphocytes [67]. The impairment of the antitumor effect observed in athymic mice in our model is consistent with the notion that TANs exert their main activity by this last mechanism. The neutrophil population has been shown to act on several levels of the adaptive response [68], including recruitment [69], antigen presentation [70], and activation of memory cytotoxic T cells [71]. However, in our model, the exact mechanisms involved in T-cell activation/stimulation by neutrophils remain to be investigated in future studies. We cannot rule out the importance of other immune cells in our model, and we have previously shown that NK cells were involved in the antitumor response induced by B16 melanoma cells transduced with p19Arf and IFNβ [41]. Neutrophils have also been shown to exert an antitumor activity by interacting with NK cells; thus, it would be interesting to evaluate whether such collaboration between these cell types occurs in our experimental model [72]. In addition, CD274 upregulation was also observed in our model, and its expression by neutrophils has been described as a mechanism of immunosuppression [73,74], suggesting that association with anti-PD1 therapy could improve the therapeutic effect of p19Arf and IFNβ gene transfer.

In conclusion, we showed that although p19Arf induced cell death, only its association with IFNβ gene transfer was able to fully promote immunogenic hallmarks *in vitro* and immune protection *in vivo,* a finding revealed for the first time in this study. Here we demonstrate a new mechanism in which the response to intratumoral immunostimulation relies on neutrophil recruitment and activity. Finally, the association of our gene transfer approach with CDDP produced a pronounced synergistic effect, indicating that manipulation of the tumor microenvironment can dramatically improve current therapies.

Conflicts of Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2016.09.011.

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