Cancer Immunol Immunother (2016) 65:371–382
DOI 10.1007/s00262-016-1807-8

ORIGINAL ARTICLE

Vaccination using melanoma cells treated with p19arf and interferon beta gene transfer in a mouse model: a novel combination for cancer immunotherapy

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Received: 28 July 2015 / Accepted: 31 January 2016 / Published online: 18 February 2016

Abstract Previously, we combined p19^{Arf} (*Cdkn2a*, tumor suppressor protein) and interferon beta (IFN-β, immunomodulatory cytokine) gene transfer in order to enhance cell death in a murine model of melanoma. Here, we present evidence of the immune response induced when B16 cells succumbing to death due to treatment with p19^{Arf} and IFN-β are applied in vaccine models. Use of dying cells for prophylactic vaccination was investigated, identifying conditions for tumor-free survival. After combined p19^{Arf} and IFN-β treatment, we observed immune rejection at the vaccine site in immune competent and nude mice with normal NK activity, but not in NOD-SCID and dexamethasone immunosuppressed mice (NK deficient). Combined treatment induced IL-15, ULBP1, FAS/APO1 and KILLER/DR5 expression, providing a mechanism for NK activation. Prophylactic vaccination protected against tumor challenge, where markedly delayed progression and leukocyte infiltration were observed. Analysis of primed lymphocytes revealed secretion of TH1-related cytokines and depletion protocols showed that both CD4$^+$ and CD8$^+$ T lymphocytes are necessary for immune protection. However, application of this prophylactic vaccine where cells were treated either with IFN-β alone or combined with p19^{Arf} conferred similar immune protection and cytokine activation, yet only the combination was associated with increased overall survival. In a therapeutic vaccine protocol, only the combination was associated with reduced tumor progression. Our results indicate that by harnessing cell death in an immunogenic context, our p19^{Arf} and IFN-β combination offers a clear advantage when both genes are included in the vaccine and warrants further development as a novel immunotherapy for melanoma.

Keywords Cell death · p19Arf · Interferon beta · Immunotherapy · Melanoma · Adenovirus

List of abbreviations

Ad	Adenovirus
Arf	Alternate reading frame
CCL	Chemokine (C–C motif) ligand
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CXCL	Chemokine (C–X–C motif) ligand
DEX	Dexamethasone
EPM-UNIFESP	Escola Paulista de Medicina-Universidade Federal de São Paulo
FAS/APO1	Fas receptor/apoptosis antigen 1
FMUSP	Faculdade de Medicina-Universidade de São Paulo
H60a	Histocompatibility 60a

Electronic supplementary material The online version of this article (doi:10.1007/s00262-016-1807-8) contains supplementary material, which is available to authorized users.

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ICESP	Instituto do Câncer do Estado de São Paulo
IU	Infectious units
KILLER/DR5	KILLER/death receptor 5
LUC	Luciferase
MDM2, 4	Murine double minute 2, 4
MOI	Multiplicity of infection
NOD-SCID	Non-obese diabetic-severe combined immunodeficiency
PG	p53-responsive promoter
PGTxβ	p53-responsive promoter
Raet 1d, e	Retinoic acid early transcript 1d, e
RGD	Argenylglycylaspartic acid
SEM	Standard error of the mean
TP53	Tumor protein 53, p53
ULBP1	UL16-binding protein 1
UNIFESP	Universidade Federal de São Paulo

Introduction

One key aspect of melanoma, in comparison with other cancers, is that p53 remains wild type in 80–95 % of the cases and this may represent an interesting therapeutic opportunity [1]. Under homeostasis conditions, p53 (TP53) levels are maintained low mainly because of its inhibitors MDM2 and MDMD4 that target p53 for proteasome-mediated degradation [2]. However, under oncogenic stress, the protein $p19^{Arf}$ ($p19^{Arf}$ for mice and $p14^{ARF}$ for humans) binds directly to MDM2 or relocates it to the nucleolus, blocking the MDM2-mediated degradation of p53. Antitumor functions of Arf are not fully elucidated, though it can act in a p53-independent manner to regulate ribosomal biogenesis, transcription, response to DNA damage, apoptosis and autophagy [3]. Deletions in the $p19^{Arf}$ gene (*Cdkn2A*) occur in wild-type p53 expressing melanomas that do not have MDM2 and MDM4 amplifications, suggesting that inactivation of p53 function can be established by either overexpression of MDM2 and MDM4 or loss of Arf [1].

Among the current immunotherapies recommended for the clinical management of patients with high-risk and advanced-stage melanoma, interferon alpha2 (IFNα2) has been indicated as an adjuvant therapy [4]. As a type I interferon, most effects of IFNα and IFN-β are believed to be immunomodulatory, causing an up-regulation of STAT-1 and an infiltration of dendritic cells (DC) and T lymphocytes into the tumor bed [5]. Moreover, it has been reported that IFNs can modulate more than 300 genes, mainly through the JAK-STAT pathway to exert apoptotic, antiangiogenic and immunomodulatory effects that are critical for immune rejection of the tumor [6, 7].

Previously, our group has developed a synthetic p53-responsive promoter, called PGTxβ, and used it to drive transgene expression in an adenoviral vector (AdPG) [8]. AdPG was then used to successfully mediate gene transfer of $p19^{Arf}$ or IFN-β cDNAs to the B16 mouse melanoma cell line (Arf deficient and p53 wild type) in an endeavor to associate cell death and antitumor immunity. Strikingly, we observed that, in vitro, cell death was significantly enhanced by the combined gene transfer ($p19^{Arf}$ plus IFN-β) when compared to the single therapies and also showed correlation with the up-regulation of transcriptional targets of p53. Furthermore, in a mouse model of in situ gene therapy, treatment of subcutaneous tumors by $p19^{Arf}$ and IFN-β combination decreased tumor progression and increased cell death in situ, thus prolonging survival [9]. Here, we aim to investigate the anti-tumor immune response elicited by the combined $p19^{Arf}$ and IFN-β treatment in mouse models of an anti-melanoma vaccine.

Materials and methods

Cell culture

The mouse melanoma cell line B16mCAR (hereafter called B16) was originated by the modification of the B16F10 cell line with forced expression of the murine coxsackie and adenovirus receptor as previously described [9]. The TM1 mouse melanoma cell line, kindly provided by Dr. Roger Chammas (FMUSP), was maintained as described [10].

Vector construction, virus production and detection of transgene expression

Construction of the p53-responsive adenoviral vectors (AdPG, non-replicating serotype 5) encoding the mouse cDNAs for $p19^{Arf}$ or IFN-β and virus production have been described previously [9]. Titration of adenoviral stocks was performed with the Adeno-X Rapid Titer Kit (Clontech) where titer yields were: AdPGLUC (2.8×10^{11} IU/mL, infectious units/milliliter), AdPGp19 (10^{11} IU/mL) and AdPGIFNβ (3.2×10^{11} IU/mL). Detection of $p19^{Arf}$ and IFN-β was performed as described in Merkel et al. [9].

Animal studies

C57Bl/6 (7 week old, female) and nude (*Foxn1*^{n}, 7 week old, female) mice were obtained from the Centro de Bioterismo, FMUSP. NOD-SCID mice (NOD/LtSz-Prkdc^{scid}, 8 week old, female) were obtained from UNIFESP. All animals were maintained in SPF conditions, with food and water *ad libitum*.

All procedures and conditions were approved in accordance with the guidelines of animal care and use by the Scientific and Ethics Committee of the Instituto do Coração, FMUSP.

Cell transduction and vaccine protocol

The vaccine protocol was divided into three steps: (1) ex vivo transduction, (2) vaccination and (3) challenge. For the first, B16 cells (1.2×10^6) were transduced with the vectors AdPGLUC (MOI 1800), AdPGp19 (MOI 900), AdPGIFNβ (MOI 900), or with the combination of AdPGp19 and AdPGIFNβ (MOI 900 for each one) in 10-cm dishes with 2 mL medium for 4 h before the addition of 8 mL of fresh medium. Cells were then incubated for 48 h, trypsinized, washed once with PBS and counted (viable and dying together). In the second step, these cells were inoculated (s.c) in the left flank (denominated hereafter as vaccine site) of naïve immunocompetent C57Bl/6 mice and 7 days after the last vaccine; in the last step, these animals were challenged with fresh B16 cells (1×10^5) inoculated (s.c) in the right flank (denominated challenge site). Additionally, mice were also vaccinated with cells that were previously transduced ex vivo with the AdPGLUC vector and killed with three cycles of freeze–thaw (B16 + LUC group). Tumor progression was accompanied and tumor volume calculated as described previously [9]. As specified in the results section, different cell quantities and vaccination regimens were also used. For the therapeutic vaccine model, naïve C57Bl/6 mice were inoculated (s.c) with fresh TM1 cells (8×10^4) in the right flank and 7 days later vaccinated in the left flank (s.c) with TM1 cells (4×10^5) that were transduced ex vivo with the vectors AdRGDPGIFNβ (MOI 500), or with the combination of AdRGDPGp19 and AdRGDPGIFNβ (MOI 500 for each one). In this case, the vectors contain an RGD tripeptide modification that enhances the efficiency of delivery and will be described elsewhere (manuscript in elaboration).

CD45 immunochemistry

Challenge tumors were collected on day 16 after challenge and embedded in tissue-freezing medium (Tissue-Tek/OCT™), cut in sections and mounted on poly(L-lysine)-coated slides (Sigma). For the immunochemistry, reaction slides were blocked with 1 % bovine serum albumin (30 min) and incubated (1 h, RT) with a purified rat anti-mouse CD45 antibody (1/100, BD Pharmingen) followed by Biotin Mouse Anti-Rat IgG2b (1/200, BD Pharmingen) and using the DAB substrate kit (BD Pharmingen). Slides were visualized by light microscopy, and five fields for each slide were randomly photographed and positive cells counted with the assistance of ImageJ software (NIH).

Cell cycle analysis

Cells were transduced as described above, and cell cycle analysis done as per Merkel et al. [9]. Briefly, after treatment cells were incubated in PBS containing propidium iodide and RNAse. After washing, cells were submitted to fluorescence measurement by flow cytometry (FACScan, Becton-Dickenson) and the cell cycle profile analyzed by CellQuest software (BD, USA).

Clonogenic assay

After transduction as described above, 1000 cells were plated in 10-cm dishes, maintained in culture for 12 days and then fixed with acetic acid/methanol 1:7 (v/v), washed once with PBS (10 mL) and then incubated with a 0.5 % crystal violet solution for 2 h.

RT-qPCR

B16 cells were transduced as described above, and after 48 h, mRNA was collected to perform qPCR analysis as described in detail in (1). All samples were tested in triplicate and analyzed by the 7500 Fast Software, version 2.05 (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used for gene expression quantification, and data are presented as fold change in expression (log 2) as compared to the non-transduced B16 condition. Primers are described in Supplemental Table 1.

Priming, cytotoxic assay and bead array

B16 cells (3×10^5) were transduced as described above and, after 48 h, injected into the footpad of C57Bl/6 mice. Ten days later, cells were collected from popliteal lymph nodes and co-cultured with fresh B16-LUC cells (stably modified to express luciferase) at different ratios (1:1, 1:10 and 1:20, B16: popliteal lymph node cells) for two days in a round-bottom 96-well plate. In order to evaluate cytokine production, the supernatant from the 1:20 condition was collected and subjected to cytometric bead array (BD Bio-sciences) on a FACSCaliber cytometer equipped with cytometric bead array software (BD Bio-sciences) according to manufacturer's instructions. The luciferase activity of the adhered cells was measured with Dual-Glo Luciferase Assay System (Promega) following the manufacturer's instructions and using a luminometer (Victor, Perkin-Elmer, USA).

Immune suppression

Immunocompetent C57Bl/6 mice were immunosuppressed with dexamethasone as described in [11]. Briefly, the mice

were injected in the right flank (s.c) with 3 or 10 mg/kg/day of dexamethasone (Roche) or PBS (Control group) for 19 consecutive days. On the 8 days of treatment, these mice were inoculated (s.c) in the left flank with B16 cells (1×10^5) transduced as described above, and on the twelfth day, peripheral blood from the LUC, LUC + 3DEX and LUC + 10DEX groups was collected by retro-orbital puncture to count white blood cells at the FMUSP.

$CD4^+$ and $CD8^+$ T lymphocyte depletion

In vivo depletion of $CD4^+$ or $CD8^+$ T cells was performed by treating (i.p) vaccinated mice with ascites containing GK1.5 or 53.6.7 rat IgG antibodies, respectively, kindly provided by Dr. Mauricio Martins Rodrigues (UNIFESP). The control group was treated with ascites containing the UF5H2 anti-carcinoembryonic antigen, IgG1 antibody (a human melanoma antigen) kindly provided by Dr. Roger Chammas (FMUSP). To deplete $CD4^+$ lymphocytes, mice were injected after the first vaccine on days 9, 11, 13 and 21 after first vaccination. To deplete $CD8^+$ lymphocytes, depletion started 2 days before vaccination and continued on days 4 and 6. Additionally, on day 16, one more injection was made to maintain the depletion. The efficacy of these protocols was superior to 96 % and confirmed in spleen cells and inguinal lymph nodes by FACS analysis using the following antibodies AcαCD3 (PE, 17A2, BD Bioscience), AcαCD4 (PECy7, RN4.5, Invitrogen) and AcαCD8 (APC, 5H10, Invitrogen).

Analysis of tumor infiltrating $CD4^+$ and $CD8^+$ T lymphocytes

C57Bl/6 mice were vaccinated (3×10^5 B16 cells, 1X) and challenged as explained above and 18 days later, challenge tumors were collected, dissociated in Liberase (35 μg/ml, Roche), and cells were analyzed by flow cytometry (FACScan, Becton–Dickenson) for the expression of CD3, CD4 and CD8 (using the antibodies described above).

Statistical analysis

Data are presented as mean ± SEM. Statistical differences between groups were indicated with p values, where $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Comparisons between two groups an unpaired t test were used. If more than two groups were compared, results were analyzed by one-way ANOVA followed by Tukey post hoc test. Statistical analysis for tumor progression curves was performed by two-way ANOVA and Bonferroni posttest. For survival or tumor-free mice, log rank Mantel–Cox test was performed, followed by Wilcoxon test. All analyses were made using the GraphPad Prism 5 software.

Results

Vaccination with cells dying due to treatment with the $p19^{Arf}$ and IFN-β combination induces a protective anti-tumor immune response

In order to investigate whether the combined gene transfer of p19Arf and IFN-β could induce an anti-tumor immune response, we developed a prophylactic vaccine tumor protocol (Fig. 1a). In this model, the B16 cell line is transduced ex vivo and while dying (when cell death is apparent upon cell cycle analysis, but has not yet reached its maximum, Fig. 1b) is used as the immunizing agent against a subsequent tumor challenge. In controls groups, mice were vaccinated with PBS, live B16 cells (to investigate the influence of a tumor at the vaccine site on the progression at the challenge site) or dead B16 cells that were transduced ex vivo with AdPGLUC vector (to control for the presence of tumor and viral antigens). As hoped, at the challenge site, only the group that received the $p19^{Arf}$ and IFN-β vaccination showed a significant reduction in tumor volume (66.5 ± 47 mm^3) when compared to control groups, such as Dead B16 + LUC (394 ± 97.03 mm^3) (Fig. 1c). Challenge tumors were collected and analyzed for the presence of the common leukocyte antigen (CD45) as a preliminary examination of the involvement of the immune system. Indeed, an increase in $CD45^+$ cells was observed only in the p19 + IFN-β challenge tumors (Fig. 1d). Despite the induction of cell death by the $p19^{Arf}$ and IFN-β combination, B16 cells still formed tumors at the vaccine site. Interestingly, tumor formation was significantly delayed (Fig. 1e) and progression significantly reduced (Supplemental Figure 1) in the p19 + IFN-β group as compared to the live B16 group.

Conditions for tumor development at the vaccine site

To investigate the possible conditions that give rise to the tumors at the vaccine site, we first performed a clonogenic assay to reveal the resistance of B16 cells to treatment. Treatment with $p19^{Arf}$ or its combination with IFN-β drastically decreased the number of colonies formed, yet resistant clones were observed (Fig. 2a). Next, we assessed the influence of the amount of cells and the number of applications used during the vaccination with the expectation of finding limits for tumor formation at the vaccine site. For this, treated B16 cells were inoculated (s.c) with different quantities (10^5 or 3×10^5) in a single injection or divided into three weekly injections (Fig. 2b). Cells treated by the p19 + IFN-β combination tend not to develop tumors when inoculated in a single injection, regardless of the amount of cells used. Yet, when the same quantity of cells was divided into three injections, tumors were formed, as observed in the 3×10^5 (1X) and 10^5 (3X) groups. Additionally, tumors

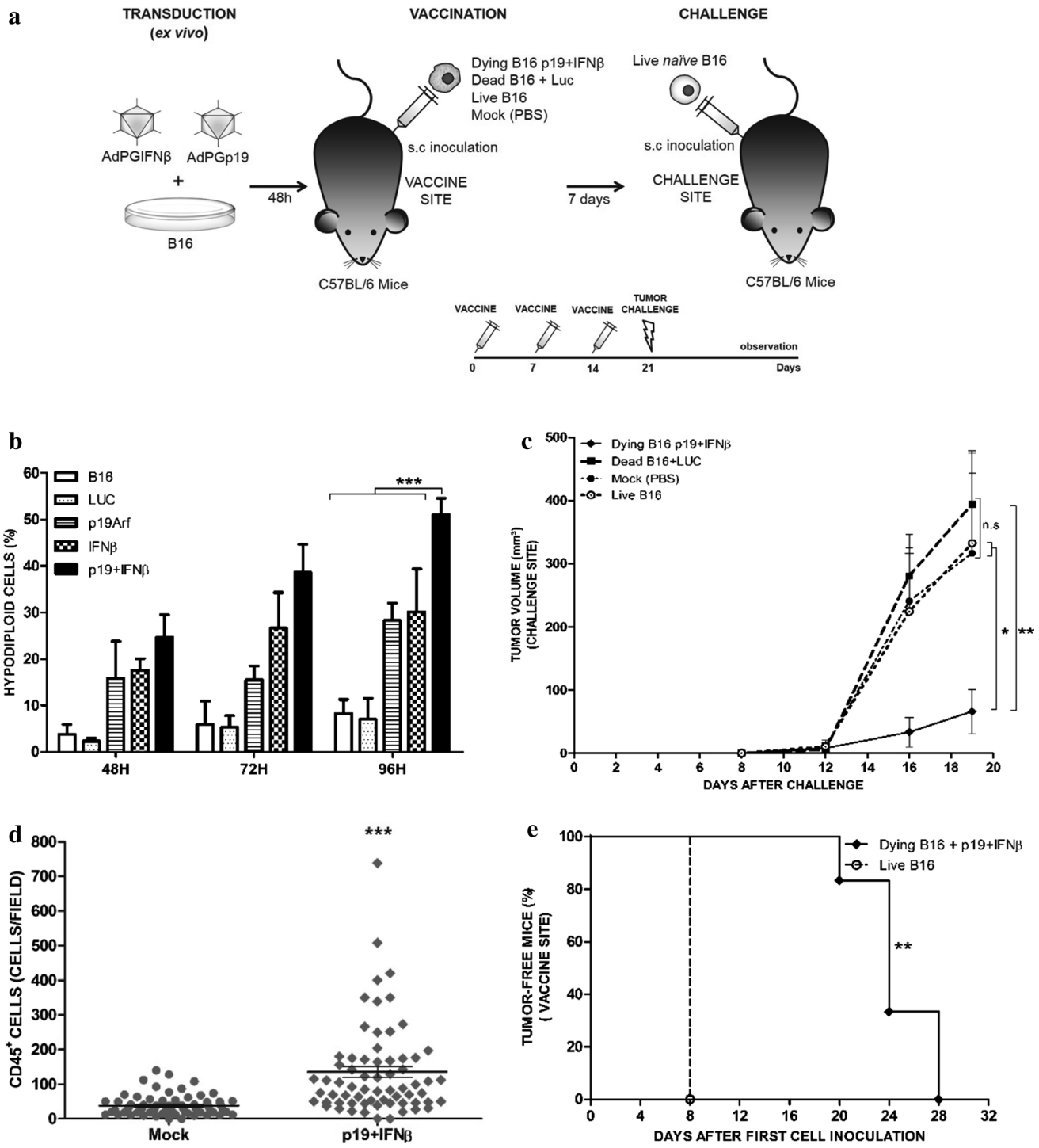

Fig. 1 Vaccination with cells dying due to treatment with the $p19^{Arf}$ and IFN-β combination induces a protective anti-tumor immune response. **a** Schematic representation of the vaccination protocol. C57Bl/6 mice are inoculated (3×, once per week) in the vaccine site with PBS, dead cells transduced with the vector AdPGLUC, live cells (only 1 application with 5×10^5 cells at day 14) or B16 cells co-transduced with the vectors AdPGp19 and AdPGIFNβ (3×10^5 cells). On day 21, the animals are challenged. **b** Cell cycle analysis of transduced B16 cells reveals kinetics of hypodiploid population in vitro. **c** Tumor progression at the challenge site. **d** Immunohistochemical analysis of $CD45^+$ infiltrating cells in the challenge tumors. **e** Tumor onset at the vaccine site. Dying B16 p19 + IFN-β ($n = 5$); Dead B16 + LUC ($n = 5$); Mock ($n = 5$); Live B16 ($n = 3$)

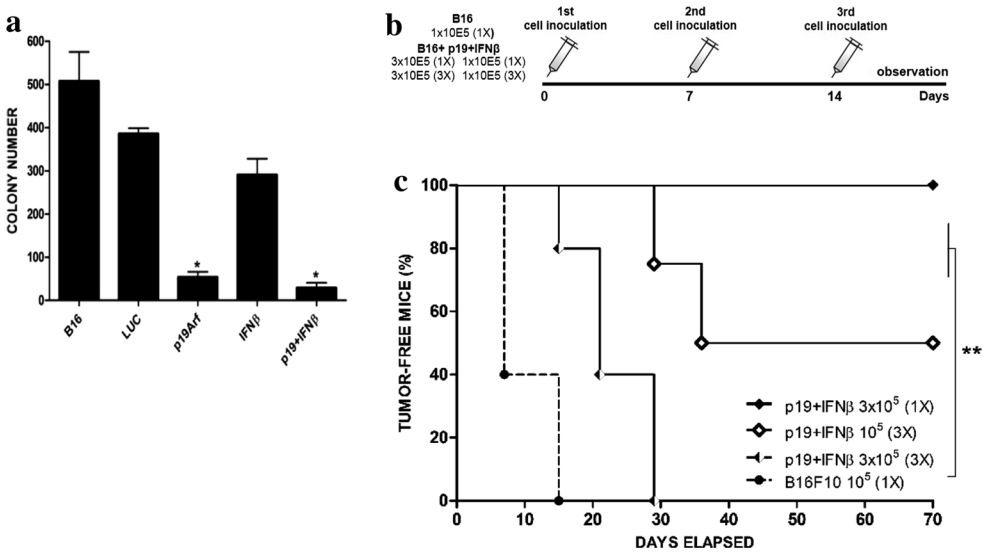

Fig. 2 Conditions for tumor development at the vaccine site. **a** Clonogenic assay of treated B16 cells. Data from three independent experiments. **b** Schematic representation of the cell inoculation protocol. B16 cells are co-transduced with the vectors AdPGp19 and AdPGIFNβ and later, animals are inoculated with 10^5 or 3×10^5 cells in a single injection or divided into three injections, one each 7 days. **c** Tumor onset in the inoculated mice. $n = 5$ for all groups

appeared faster when larger quantities of cells were applied, compare 3×10^5 (3X) and 10^5 (3X) (Fig. 2c). Thus, by altering the inoculation regimen, long-lasting tumor-free survival was achieved.

Treatment with $p19^{Arf}$ and IFN-β abrogates tumor formation in hosts with normal NK activity and up-regulates ULBP1 NK ligand, IL-15 and death receptors

We speculated that the rejection of the $p19^{Arf}$-and IFN-β-treated cells at the vaccine site would involve not only the induction of cell death, but also the immune system. To address this question, we inoculated (s.c) IFN-β- or $p19^{Arf}$- and IFN-β-treated cells in the hosts C57Bl/6 (immune competent), nude (adaptive immune deficient) and NOD-SCID (innate and adaptive immune deficient) (Fig. 3a). Strikingly, p19 + IFN-β tumors did not grow in either the C57Bl/6 or nude mice, which retain natural killer (NK) cell activity [12], but did grow in the NOD-SCID strain, known for having low NK activity [13]. The treatment of cells with just IFN-β was not able to abrogate tumor formation in any host, growing in 40–60 % of the animals of each group (Fig. 3b). In order to corroborate this observation, we immune-suppressed C57Bl/6 mice with 3 or 10 mg/kg of dexamethasone, as per Keil et al. [11] who showed that mice treated with dexamethasone were less resistant to B16 tumor formation due to the reduction in NK cell activity. Indeed, in the p19 + IFN-β combination group, immunosuppression with 10 mg/kg of dexamethasone was associated with tumor formation (60 % as compared to the immune competent condition). The dose of 3 mg/kg did not alter tumor formation in the combination group. In striking contrast, tumor formation in the IFN-β group was accelerated in all conditions tested (Fig. 3c), while immunosuppression did not have an impact on the already fast-growing AdPGLUC control (Supplemental Figure 2a). To confirm immunosuppression, white blood cells were counted, revealing a decrease in the percentage of lymphocytes and eosinophils below the reference values. Yet, the neutrophil population grew, elevating the total number of leukocytes (Supplemental Figure 2b).

Moreover, genes involved in NK cell migration (CXCL2, CCL2, CCL3, CCL4, CXCL1), activation (RAET-1E, RAET-D, ULBP-1, H60, IL-15) and cell death (KILLER/DR5, FAS/APO1) were analyzed in B16 cells treated with just p19Arf, IFN-β or the combination. A significant up-regulation of the IL-15 cytokine, ULBP1 NK ligand and both FAS/APO1 and KILLER/DR5 death receptors was observed only in the combination treatment, suggesting a mechanism for NK cell activation (Fig. 3d). The other genes investigated, including all of the chemokines, were not up-regulated after treatment (Supplemental Figure 2c). These assays indicate that cells treated by the p19 + IFN-β combination are rejected trough an NK-mediated immune response and suggests that, though rare, B16 cells escaping both the treatment and the NK cell activation can form tumors at the vaccine site.

IFN-β alone or in combination with $p19^{Arf}$ induces a TH1 immune response

We next employed the prophylactic vaccine model in order to investigate the ability of single or combined treatments to unleash a protective immune response against a tumor challenge (Fig. 4a). At the challenge site, tumor progression was markedly reduced to a similar extent with either IFN-β (104 ± 30.7 mm^3) or its combination with $p19^{Arf}$ (112 ± 25 mm^3). Ex vivo treatment with just $p19^{Arf}$ (256 ± 40.4 mm^3) conferred no significant protection as

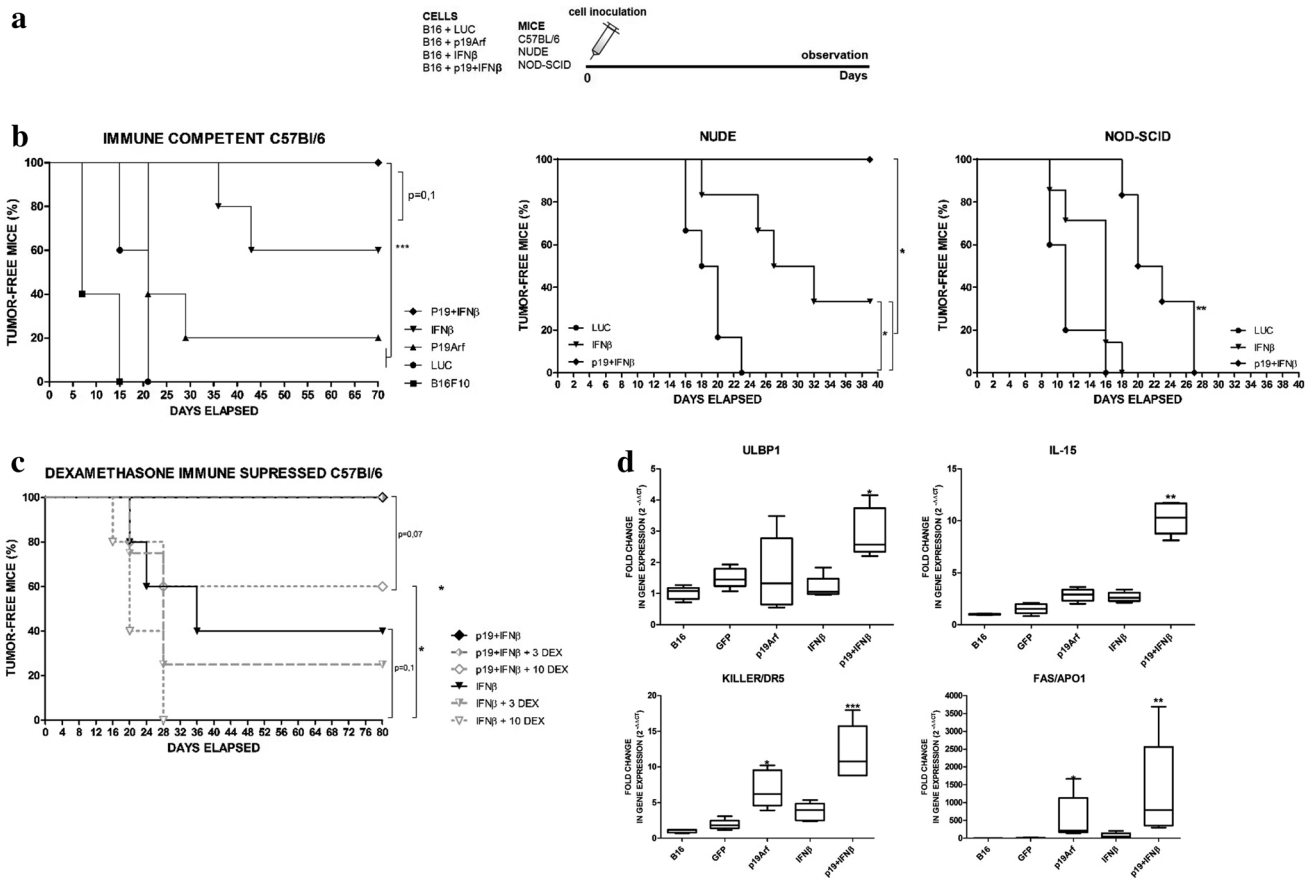

Fig. 3 Treatment with $p19^{Arf}$ and IFN-β abrogates tumor formation in hosts with normal NK activity and up-regulates ULBP1 NK ligand, IL-15 and death receptors. **a** Schematic representation of cell inoculation protocol. B16 cells transduced ex vivo with the adenoviral vectors AdPGLUC, AdPGp19, AdPGIFNβ or the AdPGp19/AdPGIFNβ combination and later inoculated (s.c) in a single application with 1×10^5 cells in C57Bl/6, nude or NOD-SCID mice. **b** Tumor onset in the inoculated mice. $n = 6$ for all groups. **c** Tumor onset in the dexamethasone immunosuppressed C57Bl/6 mice. Immune competent mice are injected (s.c) daily for 16 consecutive days with 3 or 10 mg/kg dexamethasone and on the eighth day implanted with the treated B16 cells. $n = 5$ for all groups. **d** RT-qPCR analysis of gene expression in B16 cells that had been transduced ex vivo. Cells are transduced and incubated for 48 h before collected for RT-qPCR. β-Actin is used as the reference gene. Data are calculated using $2^{-\Delta\Delta Ct}$ method and presented as fold change (log 2) as compared to the non-transduced B16 condition. Data from derived from five independent experiments

compared to the control group (586 ± 88 mm^3) (Fig. 4b). Interestingly, the p19 + IFN-β treatment was more frequently associated with tumor-free progression at the vaccine site as compared to all other conditions (Supplemental Figure 3a), thus conferring a survival benefit exclusively for this group (Fig. 4c). Alternatively, transduced B16 cells were implanted in the foot pad of C57Bl/6 mice, popliteal lymphocytes isolated and co-cultured with B16-LUC (stably modified to express luciferase) to evaluate their cytotoxic activity and cytokine expression. Bead array analysis revealed in both the IFN-β and the combination groups an increase in cytokines associated with a TH1 immune response (IL-12, IL-6, TNF-α, IFN-γ, MCP1/CCL2) (Fig. 4d), and with the exception of IL-10, no alteration was seen in the TH2 and TH17 profile (Supplemental Figure 3b). In corroboration, a significant difference in luciferase activity, used to indicate viable tumor cells, was also only observed in those groups where IFN-β was included (Fig. 4e), indicating that immune protection is dependent on IFN-β.

Both $CD4^+$ and $CD8^+$ T lymphocytes are necessary for immune protection

To uncover the role of helper ($CD3^+CD4^+$) and cytotoxic ($CD3^+CD8^+$) T lymphocytes, first we analyzed the infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in the challenge tumor 18 days after vaccination, revealing a significant increase in the percentage of both of these lymphocytes in comparison with mice in the mock treatment group (Fig. 5a, b). Moreover, depletion of $CD4^+$ or $CD8^+$ T cells was performed in the prophylactic vaccination model by

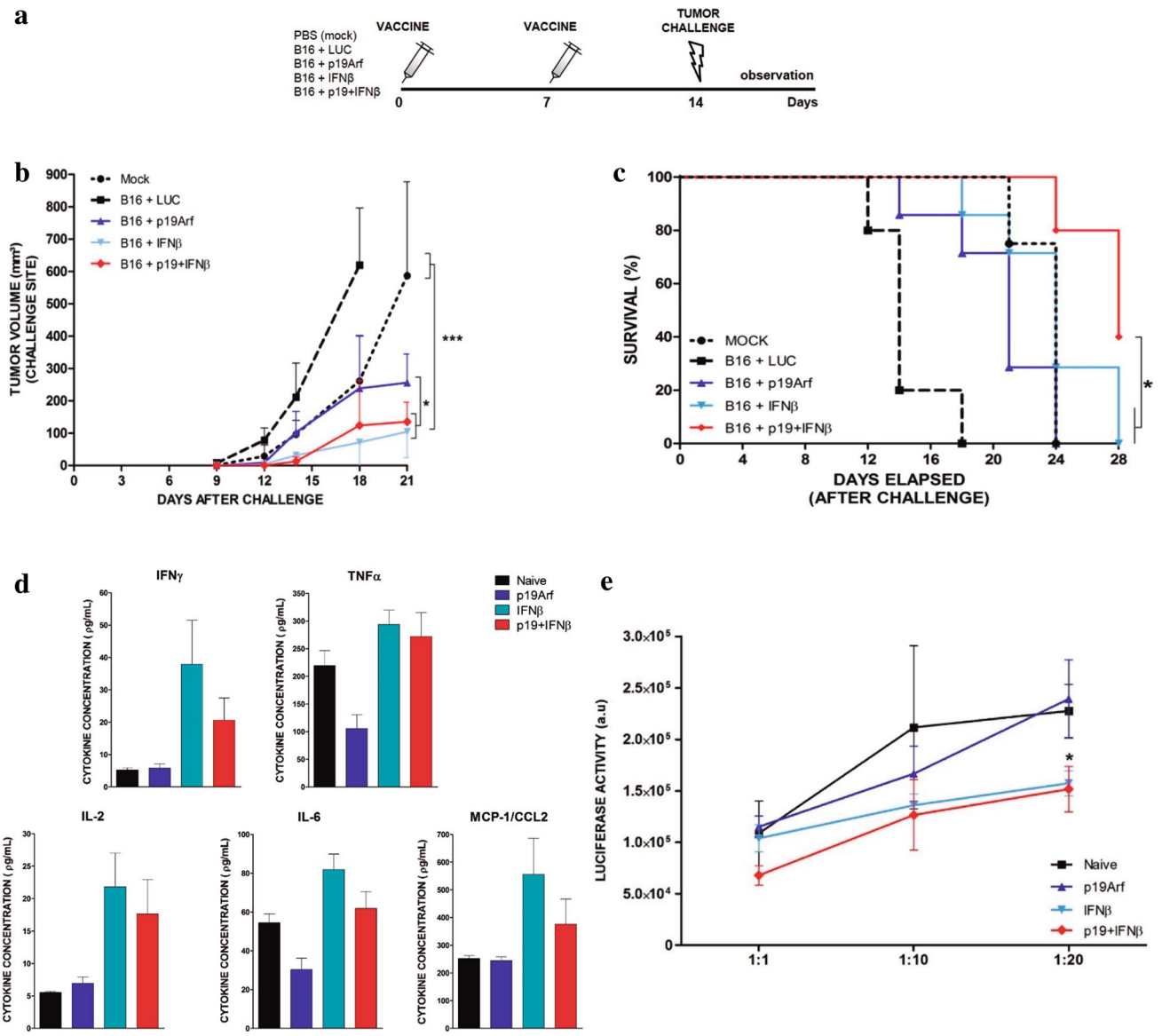

Fig. 4 IFN-β alone or in combination with $p19^{Arf}$ induces a TH1 immune response. **a** Schematic representation of the vaccination protocol. C57Bl/6 mice are vaccinated (2×, once a week) with B16 cells transduced ex vivo with AdPGLUC, AdPGp19, AdPGIFNβ or the AdPGp19/AdPGIFNβ, and on the day 14, mice are challenged. **b** Tumor progression at the challenge site. **c** Survival curve of the vaccinated mice. $n = 4$ for the mock and B16 + LUC groups. $n = 6$ for the B16 + p19 + IFN-β. $n = 7$ for the IFN-β group. **d** Cytokine expression analysis. **e** Luciferase activity of adherent B16 cells. $n = 4$ for the Mock group, $n = 5$ for the $p19^{Arf}$ and p19 + IFN-β groups and $n = 6$ for the IFN-β

injection (i.p) of monoclonal antibodies and growth of the challenge tumor was monitored. Depletion of $CD4^+$ cells after immunization revealed these cells to be critical for the protective effect, since tumors of this groups were equally as large as those in the PBS group and significantly bigger than the $p19^{Arf}$ and IFN-β groups (Fig. 5c). In sharp contrast, depletion of $CD8^+$ cells did not affect tumor growth in this approach (data not shown). However, if the depletion protocol was performed during the immunization step (not after), the loss of $CD8^+$ population was shown to be critical since the vaccine effect was completely abolished (Fig. 5d). No difference in progression at the vaccine site was noticed in either protocol (data not shown). Taken together, these results indicate that the participation of $CD4^+$ and $CD8^+$ T lymphocytes is fundamental for combating the tumor at the challenge site.

The $p19^{Arf}$ and IFN-β combination as a cancer immunotherapy strategy

The putative vaccine involving the use of cells dying due to the treatment with the combination of $p19^{Arf}$ and IFN-β

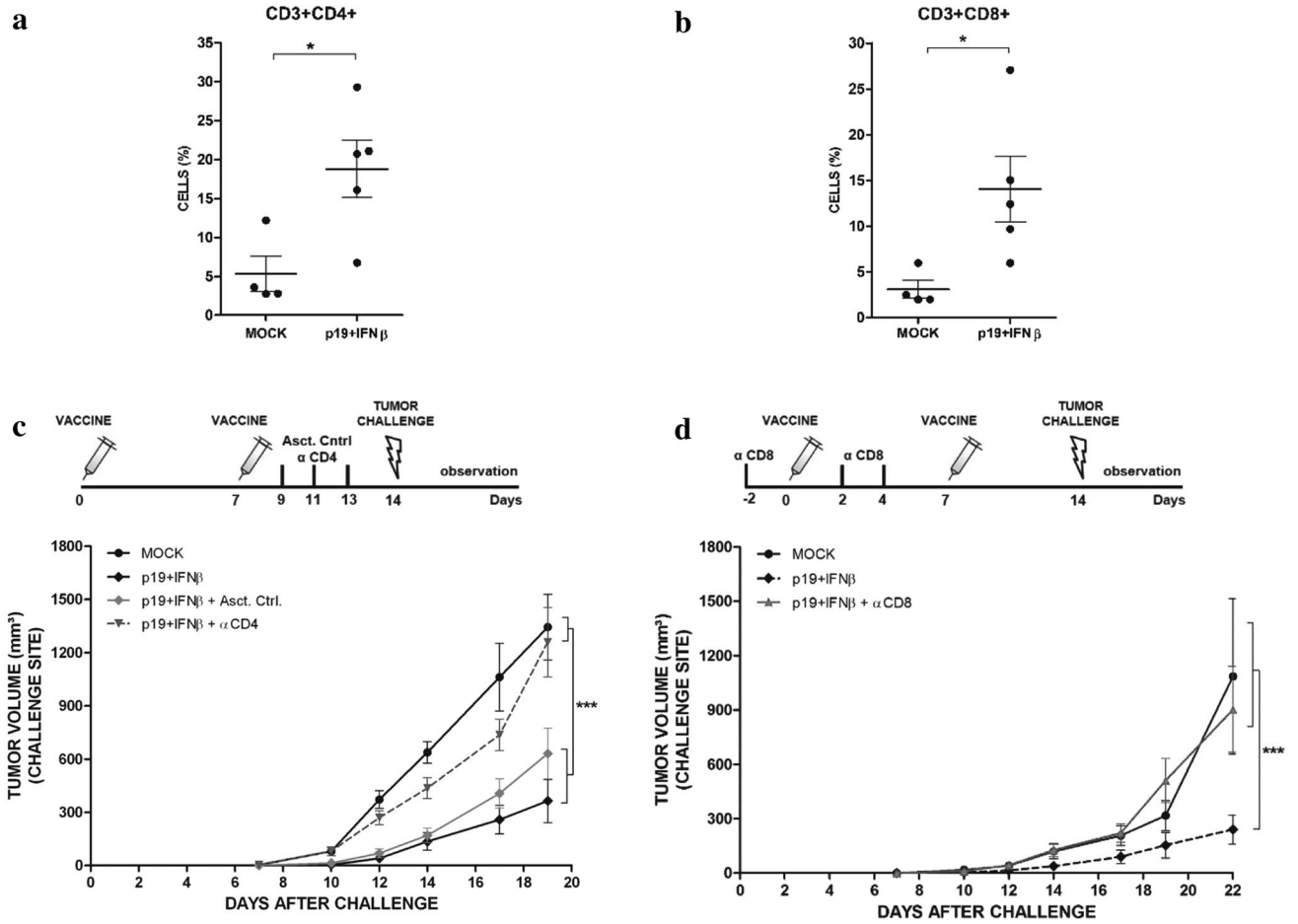

Fig. 5 Both $CD4^+$ and $CD8^+$ T lymphocytes are necessary for immune protection. Analysis of tumor infiltrating $CD3^+$ $CD4^+$ (**a**) and $CD3^+$ $CD8^+$ (**b**) lymphocytes in challenge tumors. Progression of challenge tumors upon depletion of $CD4^+$ T cells (**c**). Progression of challenge tumors upon depletion of $CD8^+$ T cells (**d**). Vaccination is made as described above. $n = 6$ for all groups

was next tested in experimental models of immunotherapy. First, we addressed the duration of protection in the prophylactic model, but with just one application of the vaccine and with the tumor challenge performed 73 days later (Fig. 6a). Challenge tumor progression was still reduced even 73 days after vaccination with 10^5 (128 ± 61.8 mm^3) or 3×10^5 cells (72 ± 39.5 mm^3), but not with 5×10^4 cells (1411 ± 328 mm^3), suggesting that an immunological memory was created and that a minimum number of cells was needed to induce protection in this setting (Fig. 6b). Next, using a different mouse melanoma cell line, called TM1, our vaccine was evaluated in a therapeutic application. To this end, mice were first inoculated with naïve TM1 tumor cells and, 7 days later, vaccinated with a single inoculation of cells treated with just IFN-β or its combination with $p19^{Arf}$ (Fig. 6c). Neither of the groups developed tumors at the vaccine site, but only the animals vaccinated with cells treated by the combination had reduced tumor progression at the challenge site (160 ± 43.3 mm^3), even when compared to the IFN-β group (316 ± 37.23 mm^3). Thus, the TM1 therapeutic vaccine model not only revealed effectiveness of our approach in a second cell line, but also indicated that the combined treatment was superior in providing immune protection in a more advanced stage of tumor progression (Fig. 6d).

Proposed model

Based upon the results presented here and in previous studies, we propose the following mechanism for this vaccine approach (Supplemental Figure 4): First, ex vivo co-transduction of B16 cells with both AdPGp19 and AdPGIFNβ adenoviral vectors reestablishes the p53 antitumor pathway and triggers apoptosis (confirmed by cleavage of caspase 3 and exposure of annexin V) [9]. Next, in the vaccination step, these dying cells also present up-regulation of the NKG2D NK ligand, IL-15 as well as cell death receptors Killer/DR5 and Fas/APO1, contributing to the activation

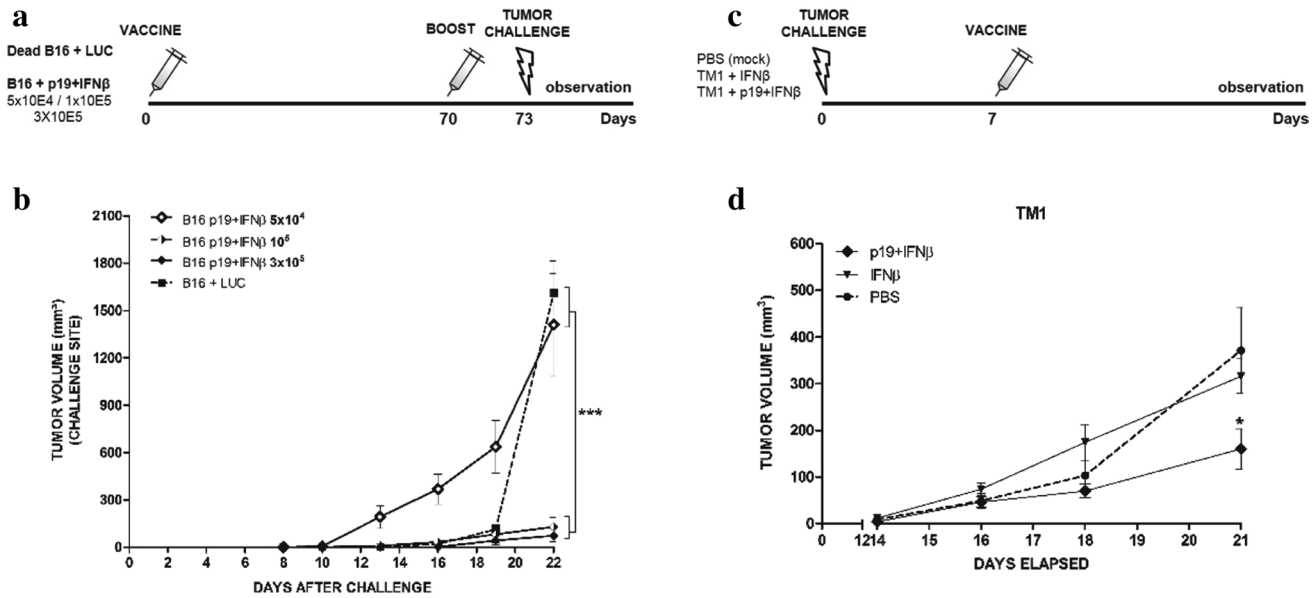

Fig. 6 The p19^{Arf} and IFN-β combination as a cancer immunotherapy strategy. **a** Schematic representation of the late challenge. C57Bl/6 mice are inoculated (single application) in the vaccine site with PBS, or B16 cells co-transduced with the vectors AdPGp19 and AdPGIFNβ (5×10^4, 1×10^5 or 5×10^5 cells). Seventy days later, a boost vaccination is made with newly treated cells (5×10^4 cells) and 3 days later (73 days since the start of the protocol) challenge with naïve B16 cells. **b** Tumor progression curve in the challenge site. $n = 4$ for the dead B16 + LUC. $n = 5$ for all the others groups. **c** Therapeutic vaccine schematic representation. On day 0, C57Bl/6 mice are inoculated with naïve TM1 tumor cells and 7 days later vaccinated with a single inoculation of TM1 cells treated with IFN-β or its combination with p19^{Arf}. **d** Tumor progression in the tumor inoculation site. $n = 6$ for all groups

of NK cells and rejection of the tumor at the vaccine site. Along with the combat of vaccine cells, we speculate that release the of cell death-associated molecules, secretion of IFN-β and exposure of tumor antigens are perceived by antigen-presenting cells of the adaptive immune system, unleashing a TH1 cytotoxic immune response. Finally, naïve tumor cells at the challenge site are attacked by $CD8^+$ and $CD4^+$ T lymphocytes that reduce tumor progression, thus prolonging survival of the mouse.

Discussion

We have shown that the vaccination using cells dying due to the treatment with p19^{Arf} and IFN-β controls outgrowth at the vaccine site and also an immune response that reduces tumor progression in both prophylactic and therapeutic models. We propose that, by occurring in vivo, the process of cell death may also contribute to the immunogenicity of our vaccine through the release of cell death-associated molecules, as demonstrated that tumor cells can die in an immunogenic cell death context [14, 15]. To be classified as an inducer of immunogenic cell death, the operational definition is: ex vivo treatment must induce prophylactic immune protection in a vaccine model and therapeutic effects (i.e., reduction in tumor growth) must depend at least in part on the immune system [14]. We believe that our results satisfy this definition since immune protection was induced by the p19^{Arf} and IFN-β vaccine, reducing tumor progression at the challenge site due to the involvement of $CD4^+$ and $CD8^+$ T lymphocytes.

Moreover, our results suggest that rejection of p19^{Arf}- and IFN-β-treated cells at the vaccine site involves NK cell activity. Additional evidence came from the dexamethasone immunosuppressed mice that developed more tumors than their immune competent counterpart. In the work of Keil and colleagues [11], dexamethasone-treated mice lost their resistance to B16 tumors due to a reduction in the lytic activity of NK cells. As in theirs, our work has also revealed a reduction in the number of lymphocytes and an increase in neutrophils, thus supporting that our suppression regime was reliable. In addition, a possible advantage of inducing NK cells at the vaccine site would be their ability to collaborate with DC to promote a TH1 immune response, thus assuming a helper phenotype [16–18].

Upon further investigation, we found that only combined treatment p19^{Arf} and IFN-β up-regulated the expression of the ULBP-1 NK ligand, the IL-15 cytokine and death receptors, factors known to influence NK response. Other studies have demonstrated a role for p53 pathway in NK-mediated immunity by up-regulating ULBP1 and ULBP2 NKG2D ligands [19, 20]. However, to the best of our

knowledge, no other work has related the up-regulation of IL-15 by p53, yet type I IFN is known to induce expression of this cytokine [21]. Interestingly, IL-15's strong immune-enhancing activity is being increasingly recognized in NK cell immunotherapy, as it stimulates survival, maturation and effector functions [22–24].

The prophylactic protection at the challenge site was more dependent on the immune modulatory effects of IFN-β than treatment with $p19^{Arf}$. We suspect that differences in the dynamics of IFN-β expression may impact the vaccine protection. The expression of IFN-β is lost when cells treated by the combination die, yet cells treated only with IFN-β survive much longer, creating an opportunity for prolonged expression and interaction with the immune system in vivo. Indeed, it has already been reported that timing and magnitude of type I interferon responses impact CD8 + T cell response [25]. Nevertheless, in the therapeutic vaccine model, only the combination brought a reduction in tumor progression, indicating that it is indeed superior to the IFN-β mono-treatment. However, it is not yet clear whether the $p19^{Arf}$ and IFN-β combination can stimulate a superior T lymphocyte response than IFN-β alone.

One possible translational scenario would be an autologous vaccine in which melanoma cells obtained from surgery could be transduced ex vivo and returned to the patient in order to activate the immune system, combatting tumor regrowth and metastasis. Though not studied here, further development, such as irradiation of the cellular vaccine, may be required to ensure the safety of this approach. The use of a vaccine, instead of in situ gene therapy, may have the added advantage of restoring tumor cell immunogenicity in a non-immunosuppressive microenvironment, thus facilitating immune recognition of treated cells, antigens and the generation of an antitumor immune response. Additional benefit may come from the association of our vaccine approach with other treatments, especially those that enhance the immune response, thus increasing efficacy. Though much work remains to be done, we believe that development of this novel immunotherapy strategy is warranted given the encouraging data that have been presented here.

Acknowledgments We are grateful for the support of the laboratories of Drs. José Eduardo Krieger (Universidade de São Paulo-USP) and Roger Chammas (Universidade de São Paulo-USP). We thank Dr. Elaine Guadelupe Rodrigues (Universidade Federal de São Paulo- UNIFESP) for helpful discussions as well as Jonatan Ersching and Dr. Mauricio Martins Rodrigues (Universidade Federal de São Paulo- UNIFESP) for assistance with antibody production. We also thank Camila Morais Melo (Faculdade de Medicina da Universidade de São Paulo-FMUSP) for assistance in the immunochemistry assay. Sao Paulo Research Foundation grants (Strauss, B.E) 13/25167-5, 11/50911-4; fellowships (Medrano, R.F.V) 13/09474-5, (Ribeiro, A.H) 11/10656-5, (Catani, J.P.P) 14/11524-3.

Compliance with ethical standards

Conflict of interest The authors have no potential conflicts of interest.

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