Nitric Oxide 64 (2017) 22–30

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Bacillus Calmette-Guerin improves local and systemic response to radiotherapy in invasive bladder cancer

Barbara Prack Mc Cormick [a, b], Denise Belgorosky [a, b], Yanina Langle [b], Natalia Balarino [a, b], Eduardo Sandes [b, 1], Ana María Eiján [b, c, *, 1]

[a] Fellowship of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[b] Universidad de Buenos Aires, Área Investigaciones, Instituto de Oncología "Ángel H Roffo", Argentina
[c] Member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

ARTICLE INFO

Article history:
Received 5 September 2016
Received in revised form
23 December 2016
Accepted 20 January 2017
Available online 23 January 2017

Keywords:
Invasive bladder cancer
Bacillus Calmette-Guerin (BCG)
Ionizing radiation
Radiosensitizer
In vivo-vaccine

ABSTRACT

Background: A key factor contributing to radio-resistance in conservative invasive bladder cancer (BCa) treatment is tumor hypoxia and a strategy to overcome it is to trigger the production of nitric oxide (NO). On the other hand, ionizing radiation (IR) applied to a primary tumor can induce immunogenic cell death which may set off a cytotoxic immune response against the primary tumor and its metastasis.
Purpose: To study *in vitro* and *in vivo*, the role of BCG as a local sensitizer to overcome hypoxia-associated radio-resistance through the production of NO, and as an immune-stimulator to be used in combination with IR to generate a systemic response for invasive BCa treatment.
Materials and methods: We selected the invasive murine BCa cell line MB49-I which expresses inducible NO synthase and produces NO, cultured *in vitro* in 2D and 3D models, and inoculated *in vivo* in the subcutaneous of syngeneic mice.
Results: in vitro, multicellular murine invasive spheroids mimicked *in vivo* central tumor necrosis. BCG pre-treatment radio-sensitized spheroids through the induction of NO production, while no effect was shown in monolayers. *In vivo*, not only did BCG improve the local response to IR but it also decreased the metastatic spread and promoted the development of abscopal effect/rejection of a second tumor.
Conclusion: Since BCG has already and successfully been used for the treatment of non-invasive BCa and it improves the response to ionizing radiation in invasive BCa, these results are translational relevant to be analyzed in patients with this pathology.

1. Introduction

Urothelial carcinoma is the most prevalent bladder cancer (BCa) and ranks fourth in malignant cancer frequency in men in developed countries [1]. Although the majority of patients present non-invasive disease, 10–20% eventually progress to muscle-invasion. In addition, nearly 30% of new cases have muscle-invasion at the time of diagnosis [2].

Radical cystectomy is the gold standard treatment for patients with invasive BCa but it is associated with high complication rates and compromises patient life quality [3]. Given this, the interest to develop therapies aimed to preserve the organ, has increased. The main treatment for bladder preservation includes complete transurethral tumor resection followed by chemotherapy (CT) and radiotherapy (RT) [4].

Radio-resistance to ionizing radiation (IR) and subsequent tumor recurrence are significant problems in cancer therapy. A key factor contributing to radio-resistance is tumor hypoxia [5]. In BCa, the average hypoxic fraction reaches 10% of the tumor mass and, patients who exhibit high levels of hypoxic markers have less overall survival [6]. A strategy for tumor specific radio-sensitization in hypoxic microenvironment of solid tumors is to trigger the production of nitric oxide (NO). The inducible isoform of NO synthase (iNOS) is expressed by almost 50% of BCa patients [7] and may be induced to generate high rates of NO [8]. Local NO production

Abbreviation: listBladder Cancer, BCa; Chemotherapy, CT; Ionizing radiation, IR; Nitric oxide, NO; Inducible nitric oxide synthase, iNOS; Bacillus Calmette-Guerin, BCG; $N\omega$-Nitro-L-arginine methyl ester, L-NAME.

* Corresponding author. Área Investigaciones, Instituto de Oncología "Ángel H. Roffo", Av. San Martín 5481, C 1417 DTB, Buenos Aires, Argentina.
E-mail addresses: grupoeijan@gmail.com, anamariaeijan@gmail.com.ar (A.M. Eiján).

[1] Shared the project management.

http://dx.doi.org/10.1016/j.niox.2017.01.008
1089-8603/

induces efficient hypoxic tumor cell radio-sensitization [9] through the modulation of tumor blood flow, cellular respiration, cell signaling, and the production of reactive oxygen and nitrogen species [10].

On the other hand, IR applied to a primary tumor can induce immunogenic cell death, which may set off a cytotoxic immune response against the primary tumor and its metastasis. This phenomenon is known as abscopal effect [11,12] and it can be stimulated by the local administration of exogenous pathogenic signals [11].

Bacillus Calmette-Guerin (BCG) is the standard treatment for patients with non-muscle invasive high histological grade BCa and this therapy has shown to be the most successful immunotherapy used against any human neoplasm [13,14]. Besides, BCG stimulates the local production of NO in the bladder due to the induction of iNOS expression in urothelial cells, whereas no evidence of systemic NO formation has been noted [15].

For these reasons, in this study we analyzed BCG as a local sensitizer to overcome hypoxia-associated radio-resistance and as an immune-stimulator to be used in combination with IR to generate a systemic immune response for invasive BCa treatment. To test this hypothesis, we used the invasive murine BCa cell line MB49-I which expresses iNOS and produces NO [16] making this pre-clinical model useful to study the effects of NO production in IR treatment.

2. Materials and methods

2.1. Cell culture

Murine invasive BCa cell line MB49-I [17] was maintained in RPMI-1640 medium (Gibco 31800-14) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Internegocios S.A.) and 80 μg/ml gentamycin, at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. BCG

Living BCG (Pasteur1172 P2 strain-3x10^6 CFU/mg/ml-) suspensions were obtained from ANLISCG, Malbrán-Argentina. This strain is the one that is currently being used in our country for human bladder cancer therapy.

2.3. Irradiation

Monolayers, spheroids and tumors were irradiated using a Theratron 780 with ^{60}Co source gamma radiation emitter, with a dose rate of 1 Gy/min.

2.4. Multicellular tumor spheroids generation

1×10^4 cells were seeded on the cover of 48-well plates in 20 μl drops. Covers were inverted and incubated for 72hs until spheroids were fully formed, after which they were transferred into individual wells coated with 1.5% agarose and 500 μl of complete medium. Medium was refreshed twice a week. Experiments were performed when spheroids reached a diameter≥550 μm.

2.5. Cell viability

8×10^3 cells were plated in 96-well plates cultured 24 hs and then treated with BCG 0, 0.5, 1 and 2 mg/ml, in RPMI with 2% FBS, corresponding to 9.4 BCG/cell, 18.8 BCG/cell and 37 BCG/cell, respectively. After 24hs viability was measured at 492 nm using the colorimetric MTS assay (Cell Titer 96®AQ_{ueous}Promega, G5421) in a Multiscan Ascent microplate.

2.6. Nitric oxide determination

MB49-I sub-confluent monolayers or spheroids were treated with BCG (0, 0.5, 1 and 2 mg/ml) in RPMI with 2% FBS, with or without 2 mM Nω-Nitro-L-arginine methyl ester (L-NAME). NO was determined in culture supernatant 24hs later using the Griess reagent, as described [18].

2.7. Clonogenic assays

To assess radio-sensitivity, MB49-I cells from treated monolayers (500 cells/well for 0 and 2Gy, 750 cells/well for 4Gy and 1500 cells/well for 6Gy), spheroids (1000 cells/well) or *in vivo* treated tumors (500 cells/well) were plated in 6-well plates with complete medium. Plates were incubated at 37 °C for 10–14 days until colonies were visible to naked eye. Colonies were fixed with methanol/acetic (3:1) and stained with Giemsa. Colonies with more than 50 cells were registered. Plating efficiency (PE) and surviving fractions (SF) were calculated as: *PE = mean colony number/cells plated*; and *SF = sample PE/PE of its non-irradiated control*.

2.8. Heterotopic tumor growth

C57BL/6 J mice were subcutaneously injected with 2×10^5 MB49-I cells into the flank. When tumor volume reached 50 mm^3, animals were randomly divided into four groups of five mice each: control, BCG, IR or BCG + IR. IR (18Gy) was administered in six fractions of 3Gy, being two fractions/week. For IR, mice were anesthetized with ketamine (70 mg/kg) and xylazine (5 mg/kg) and placed on paraffin stretchers with acrylic lids where only tumors remained in the irradiation field. 0.2 mg BCG dissolved in 0.1 ml of saline solution was injected intratumoral once a week. Control and IR groups received saline solution. Tumor volume was calculated as $0.4xwidth^2xlength$. Tumors were weighed and used for clonogenic assays and molecular analysis. Lungs were extracted and fixed in Bouin solution for metastasis quantification. To evaluate the abscopal effect, primary tumors of each group were resected, and five days later, mice were challenged with a second MB49-I injection in the opposite flank of the first tumor. An additional group of mice receiving IR in the tumor site and BCG subcutaneously injected far from the tumor was added.

Mice were obtained from our Institute's animal facility and handled in accordance with the international procedure for Care and Use of Laboratory Animals. Protocols were approved by the Institutional Review Board CICUAL, protocol number 2012/02, “Instituto de Oncología Ángel H Roffo”.

2.9. Immunobloting

Proteins (80 μg) from whole-cell extracts coming from *in vivo* tumors were electrophoresed on SDS–PAGE and transferred to PVDF membranes as previously [19]. Immune-detections were performed with a procaspase-3 antibody (sc-7148). Densitometry units were referred to β-actin (Sigma A5441).

2.10. Histological study

Hematoxylin and eosin (H&E) staining for histological diagnosis were performed in paraffin-embedded sections of MB49-I spheroids as previously [19]. Zhiel-Neelsen staining was used to evaluate presence of BCG; paraffin-embedded sections of treated spheroids were incubated in 100% of fucsin for 1 h and washed with bleach (3% HCl in 95% ethyl alcohol) until discoloration, followed by a

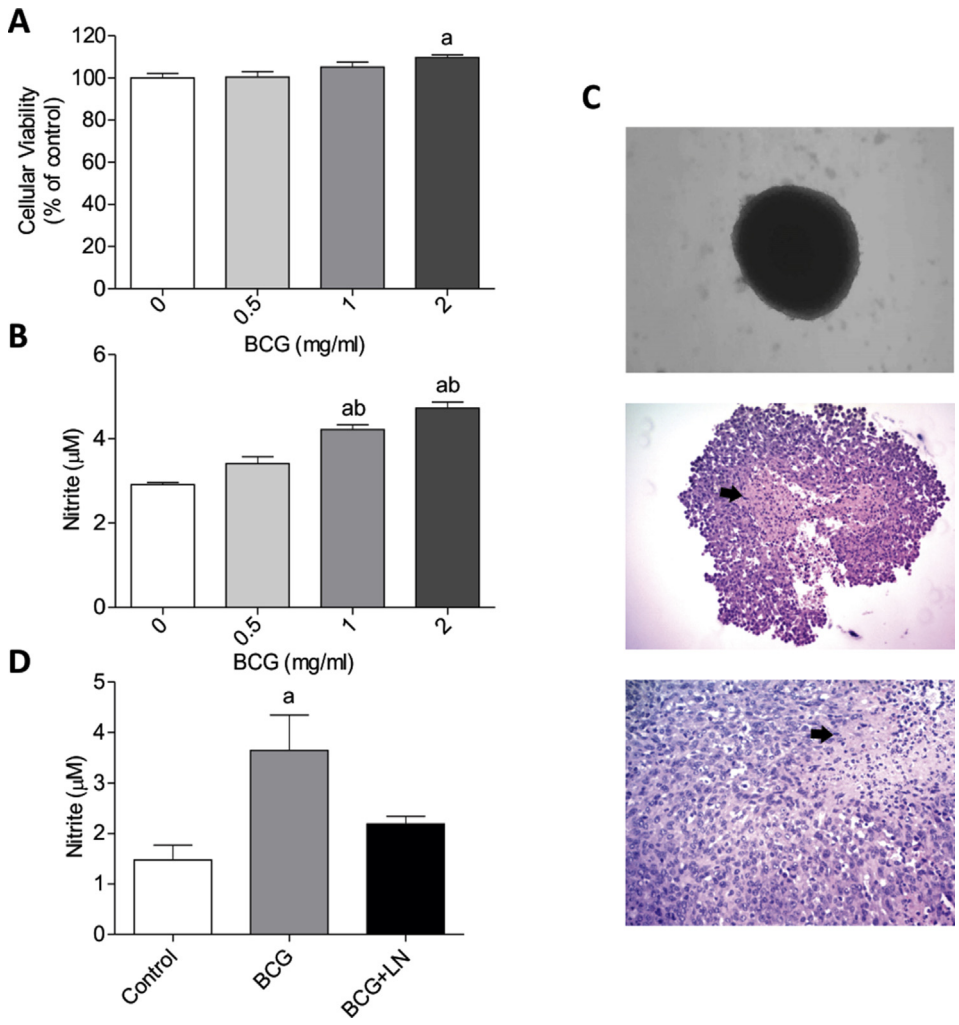


Fig. 1. BCG induced the production of NO in MB49-I cells, both in monolayers and cultured in multicellular spheroids. A, and **B**, cells in monolayers treated with increasing concentrations of BCG (0, 0.5, 1 and 2 mg/ml). **A**, MTS assays were performed to evaluate the influence of BCG treatment on cell viability. **B**, Griess assays were employed as an indirect method to assess NO levels as nitrite in response to BCG treatment. Nitrite levels were measured in conditioned media coming from treated monolayers. ANOVA Test with Bonferroni post-test, a = b = $p < 0.01$, "a" in comparison to control, "b" in comparison to BCG 0.5 mg/ml. **C**, 3D culture model by multicellular spheroids formation; *upper,* 15 days old spheroid (magnification = 40x), *middle,* histological section of a 15 days old spheroid stained with hematoxylin and eosin (magnification = 100x), and *bottom,* histological section of a MB49-I tumor stained with hematoxylin and eosin (magnification = 200x); black arrows point central necrotic areas.**D**, 15 days old MB49-I spheroids were treated with BCG 1 mg/ml and nitrite levels were measured in conditioned media. NOS pharmacological inhibitor L-NAME was employed. ANOVA Test with Bonferroni post-test, a = $p < 0.05$ in comparison to control. *Columns*: mean, *bars*: standard deviation.

staining with methylene blue during 20 min, as previously described [20]. Immunohistochemistry assay was performed as previously [19], to evaluate iNOS and gamma-histone H2AX (γ-H2AX) expression in paraffin-embedded sections of MB49-I spheroids. Specific antibodies to iNOS (abcam15323, 1:100 dilution) and γ-H2Ax (phospho S139) (ab11174, 1:5000 dilution) were used. Labelled streptavidin biotin (LSAB; Dako Cytomation, CA), at first biotinylated link universal was applied.

2.11. Statistical analysis

All experiments were done in triplicate and were repeated at least twice. Results are expressed as mean and SD or as median and range. Statistically significant values were compared using ANOVA and Bonferroni's contrast or Kruskal–Wallis and Dunn's post-test, using the 5.0 Graph-Pad-Prism statistical package; p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Multicellular spheroids mimic in vivo central tumor necrosis and the response to BCG treatment

To assess the radio-sensitizing activity of BCG in invasive BCa, we proposed a 3D culture method based on spheroids. One of the advantages of this model is the resemblance to the *in vivo* tumor growth and its hypoxic conditions. We demonstrated that MB49-I cells form multicellular spheroids with a central necrotic area, similar to those observed in MB49-I tumors (Fig. 1C).

BCG treatment effects depended on the study model. Monolayers treated 24hs with 2 mg/ml BCG showed a slight increase in cell viability (Figure 1A) and 1 mg/ml BCG increased clonogenic survival (Fig. 3A). Conversely, no effect on clonogenic survival was observed when BCG was administered to spheroids (Fig. 3C). The presence of BCG inside of spheroids was detected by Ziehl-neelsen staining, reinforcing the importance of the 3D model (Fig. 2A).

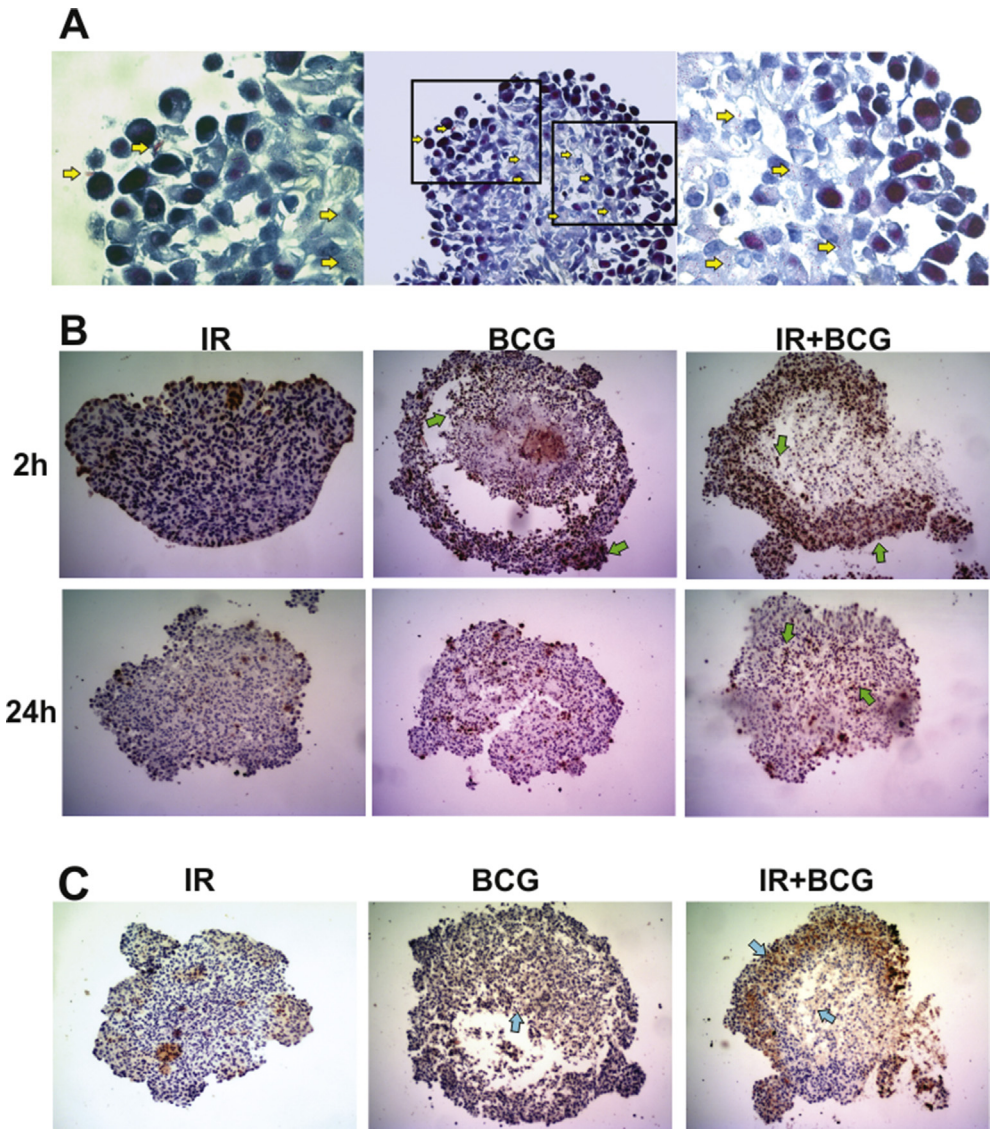


Fig. 2. BCG induced iNOS expression, and the combined treatment with IR induced an increase of γ-H2AX in spheroids. A, The presence of BCG inside of the spheroids was determined by Ziehl-Neelsen stain. Yellow arrows indicate intracellular bacilli, visualized in pink color (magnification 100x in the central picture and 1000x in the sides). ***B***, Immunohistochemistry for γ-H2AX in spheroids treated with BCG (1 mg/ml), IR (4Gy) or the combination after 2 h and 24 h of IR. Green arrows indicates positive expression (magnification 100x). ***C***, Immunohistochemistry for iNOS. Light blue arrows indicate positive expression (magnification 100x). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. BCG induces NO production in monolayers and spheroids

To further assess if BCG could sensitize MB49-I cells to IR, we first tested its ability to induce NO production. We detected increased NO levels in MB49-I monolayers and spheroids treated with BCG (Fig. 1B and D). Furthermore, this NO increase in spheroids was reverted by treatment with the NOS inhibitor L-NAME (Fig. 1D). The high iNOS expression was evident in spheroids treated with BCG and BCG + IR (Fig. 2C).

3.3. BCG sensitizes MB49-I spheroids to IR

Although MB49-I growing in monolayers as well as spheroids produced increased levels of NO in response to BCG, the sensitivity to IR was dependent on the culture model. Clonogenic assays revealed no radio-sensitivity effect of BCG on cells growing in monolayers (Fig. 3B). Conversely, BCG sensitized MB49-I spheroids to IR. Furthermore, BCG radio-sensitizing effect was dependent on NO generation, since this effect was reverted in the presence of L-NAME (Fig. 3C). To understand more about the mechanism by which BCG radio-sensitizes MB49-I spheroids we measured the expression of γ-H2AX as an indicator of DNA double strand break, as a marker of severe genomic DNA damage. Our results show that BCG increased DNA double strand breaks in response to IR, given that γ-H2AX expression was higher in spheroids treated with BCG + IR in comparison to the ones that received only IR (Fig. 2B). Analyzing the kinetic of expression, we observed a high expression 2 h post IR that persisted, although in lower levels, after 24 h indicating unrepaired DNA damage.

3.4. IR treatment reduces MB49-I tumor growth

To analyze the *in vivo* BCG capability to improve MB49-I tumors response to IR, we followed up the tumor growth. As a measure of

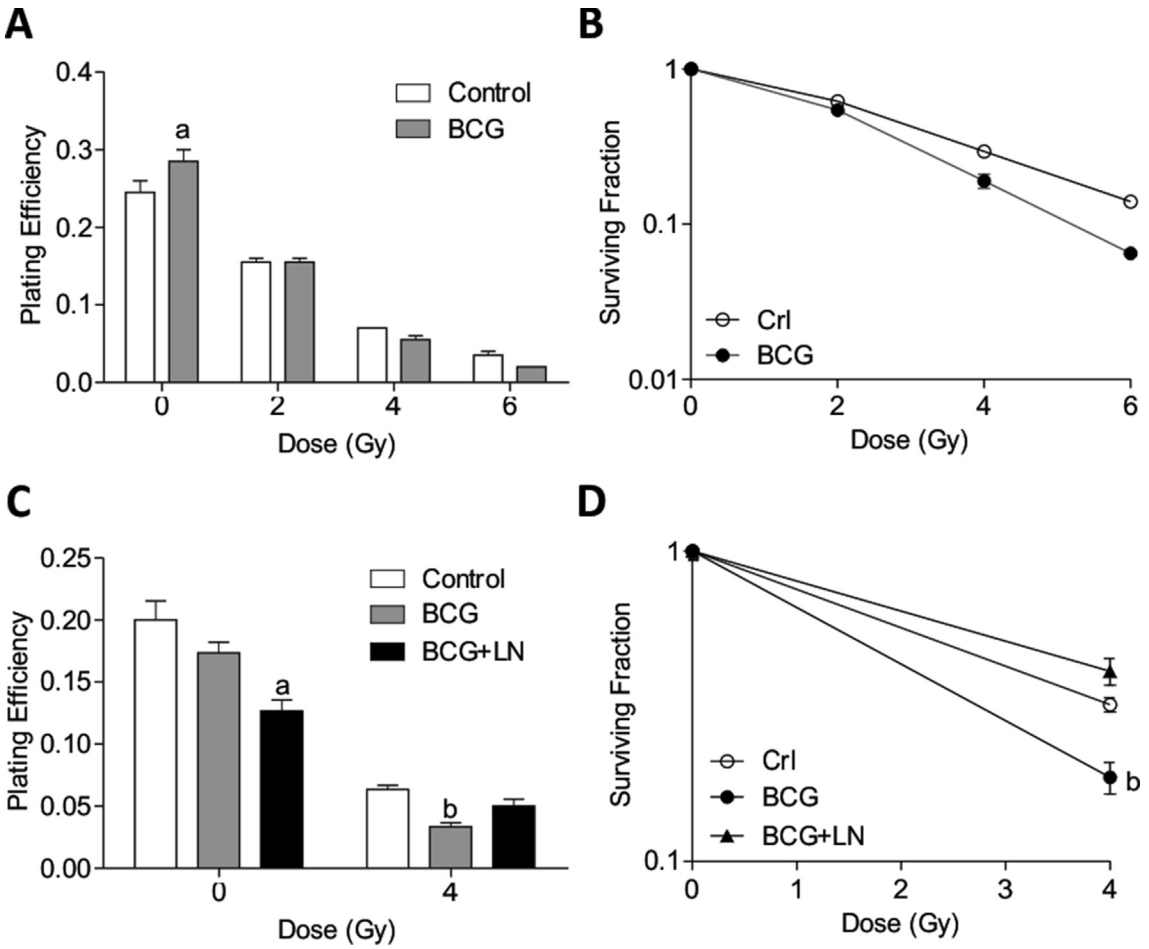


Fig. 3. BCG radiosensitized MB49-I cells cultured in spheroids, while it did not have any effect on sensitivity of cells growing in monolayer. Furthermore, BCG radiosensitizing effect was dependent on NO levels. Clonogenic assays were performed to assess cell survival after BCG and IR treatments. **A**, platting efficiency and **B**, surviving fraction obtained from clonogenic assays performed with cells from monolayers treated with BCG 1 mg/ml and IR. ANOVA of two ways (interaction $p < 0.05$) and Bonferroni's post-test, a = $p < 0.05$ difference between control and BCG when IR = 0Gy. **C**, platting efficiency and **D**, surviving fraction obtained from clonogenic assays performed with cells from spheroids treated with BCG 1 mg/ml, an inhibitor of NO synthesis (L-NAME) and IR. ANOVA of two ways (interaction $p < 0.01$) and Bonferroni's post-test, a = $p < 0.001$ difference between control and BCG + L-NAME when IR = 0Gy, b = $p < 0.05$ difference between control and BCG when IR = 4 Gy. *Columns*: mean, *bars*: standard deviation.

response, we assessed tumor volume along the assay and final tumor weight. Tumor growth curves showed that both IR and BCG + IR treatments significantly improved the antitumor response as compared to control. IR treatment stalled tumor growth but at the end of the treatment tumors started growing again, suggesting the appearance of radio-resistant cells. On the other hand, the combined treatment with BCG made MB49-I tumors regress from day 13 until the end of the experiment (Fig. 4B).

Results gathered from final tumor weight supported this evidence, since tumors from BCG + IR mice had the lowest weight and the best response to therapy (Fig. 4C).

3.5. BCG sensitizes MB49-I tumors to IR

To further analyze and comprehend the trends observed between IR and BCG + IR, we studied the surviving fraction of tumor cells and the activation of a possible death pathway, once *in vivo* treatments were finished.

On day 25 after challenge, we extracted the remnant tumors, disaggregated them, and platted living cells to evaluate the surviving fraction. Those clones, which conserve their proliferative capacity after treatment, are usually the ones responsible for the recurrences observed in patients. IR decreased tumor growth (Fig. 4B), and decreased mildly, but not significantly the number of proliferative clones. A significant effect was only reached when IR was combined with BCG (Fig. 5B), supporting the results observed *in vitro* (Fig. 3C and D).

Given the tumor regression observed in BCG + IR treated mice (Fig. 4B) we assess the activation of caspase-3 in each of the remnant tumors extracted. BCG + IR combination increased caspase-3 activation, while this activation was less evident in the other treatments (Fig. 5C).

3.6. The combined treatment decreases in vivo metastatic spread

In parallel to the residual tumor analysis, we followed up tumor-resected mice in order to assess progression to metastatic disease. On day 44 after challenge, mice were euthanized, and lungs were fixed to quantify presence and volume of metastasis.

All mice coming from control group had succumbed to the metastatic spread at day 44. Due to the high number of metastasis in control lungs, we could only calculate its number in two of them, giving more evidence about the aggressive phenotype of MB49-I tumors (Fig. 5E).

IR treatment showed no effect on metastatic spread (Fig. 5D and E). However, not only did the combination with BCG decrease the

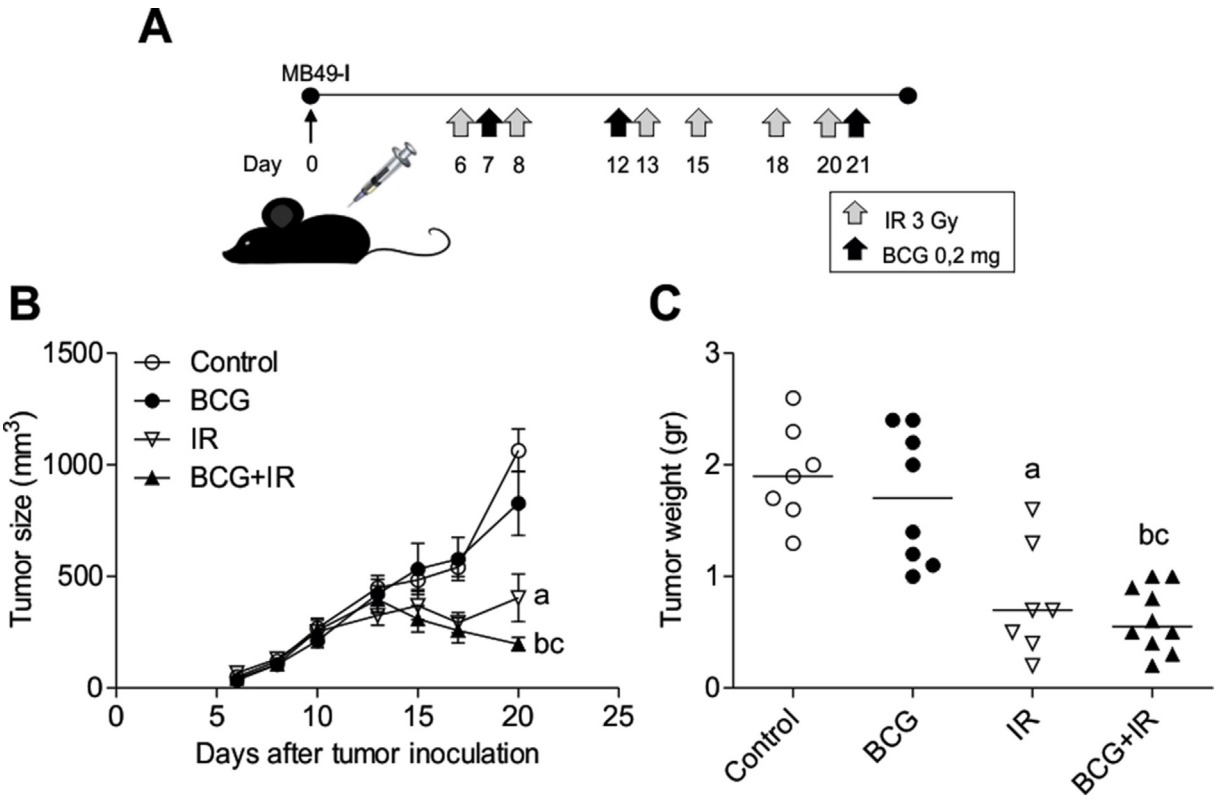


Fig. 4. IR treatment reduced MB49-I tumor growth, while the combined treatment made MB49-I tumors regress. C57BL/6 J mice were injected with MB49-I cells subcutaneously into the left flank and tumors were allowed to grow. Animals were randomly divided into four groups of five mice each: control, BCG, IR or BCG + IR. BCG treatment (0.2 mg in 100 μL of saline solution) was given as an intratumor injection. IR was administered in fractionated doses of 3Gy, 2 days/week reaching a total dose of 18 Gy. **A**, experimental design. **B**, effect of treatments on tumor growth *in vivo*. **C**, effect of treatments on tumor weight at the end of the experiment. **B** and **C,** Kruskal Wallis Test (**B**: $p < 0.01$, **C**: $p < 0.001$) with Dunn's multiple comparisons, a = $p < 0.05$ and b = $p < 0.01$ difference with control, and c: $p < 0.05$ differences with BCG.

volume of lung metastasis (Fig. 5D), but it also diminished the percentage of mice with lung metastasis (Fig. 5E).

3.7. BCG treatment improves abscopal effect to IR

Tumor-bearing mice treated and subsequently operated to remove the tumor were subjected to tumor re-challenge in the opposite flank to assess the abscopal effect (Fig. 6A). Since all mice from control group succumbed to the metastatic spread caused by the primary tumor, we could not re-challenge them. Second tumor size was lower in mice treated with IR compared to naive (Fig. 6C); however, all animals developed tumor (Fig. 6B). Regarding BCG alone, 40% rejected second tumor growth (Fig. 6B). BCG + IR significantly increased tumor growth protection (Fig. 6C). In this group (n = 9), 56% of mice rejected the second tumor growth (Fig. 6B), suggesting that BCG promotes an anti-tumor immunological memory. Similar results were obtained in an additional group of mice receiving IR in the tumor site and BCG subcutaneously far from the primary tumor, suggesting a systemic effect of BCG that culminates in both, a local and distant effect when it is combined with IR (data not shown).

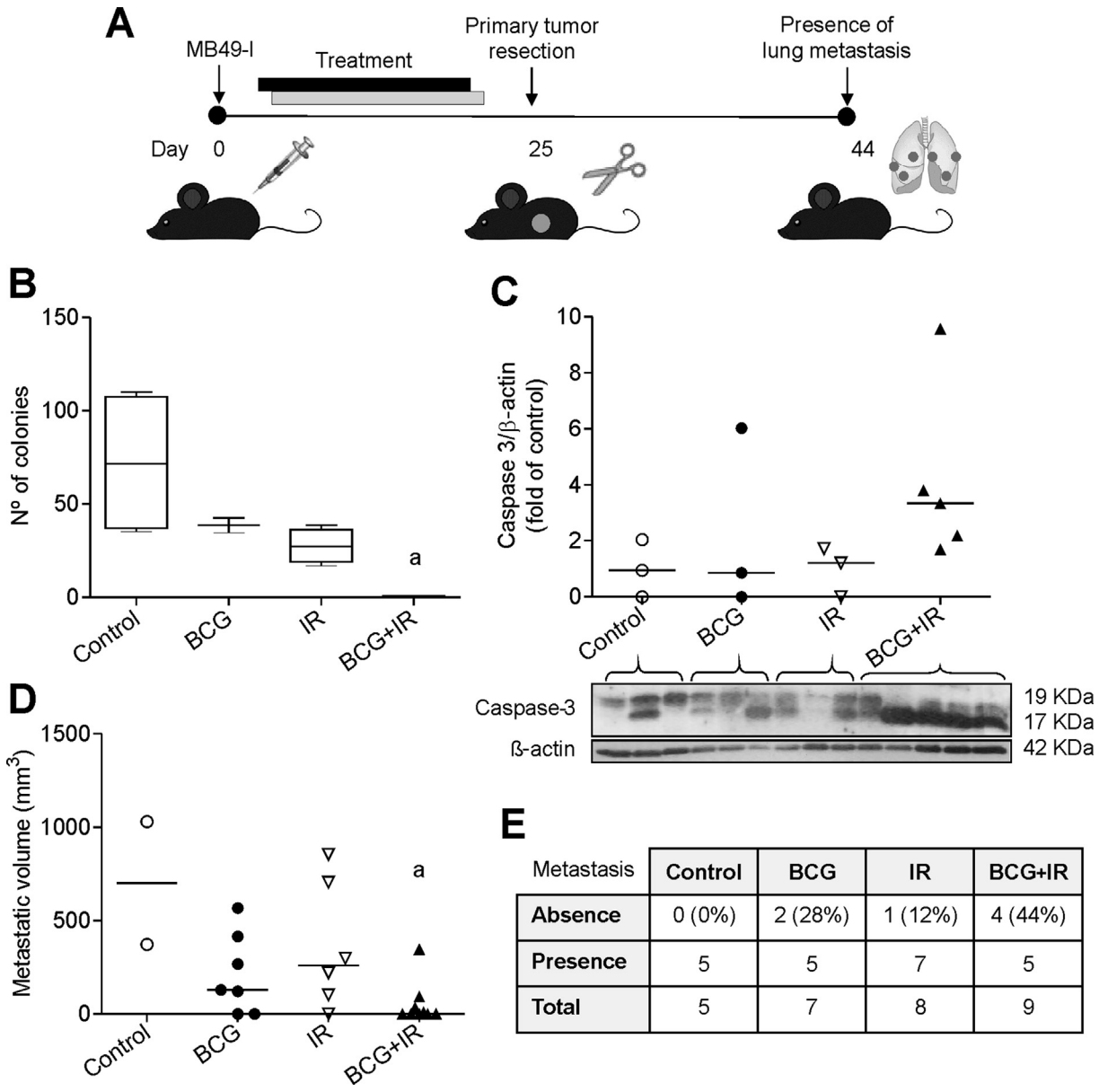
4. Discussion

Tumor hypoxia is a key factor contributing to radio-resistance [5] and it is associated with less overall survival in patients with BCa [6]. It has been reported that NO is an efficient hypoxic radiosensitizer [10] and that BCG induces the local production of this free radical in the bladder due to the induction of iNOS [15]. Considering this, we analyzed BCG as a potential radio-sensitizer in murine MB49-I BCa cells.

In this study, we showed that the response of BCa cells to BCG treatment depends on the model of study. When cells growing in monolayers were treated with BCG, we observed a slight increase in cell viability and in clonogenic survival, while no radio-sensitizing effect was detected. In multicellular spheroids, BCG showed no effect on clonogenic survival; however, when it was combined with IR it sensitized MB49-I cells. Similar results were achieved from *in vivo* tumor growth where BCG improved IR-response, supporting our spheroid model.

In our 3D model, as its mechanism of action, BCG was internalized by the cells inside the spheroid and induced the expression of iNOS enzyme. In agreement with our hypothesis, BCG radiosensitizing activity was, at least in part, a consequence of NO production through NOS activity, since the NOS inhibitor L-NAME reversed such sensitivity observed. It has been proposed that NO can bind to sites of DNA breaks preventing DNA repair and in consequence augmenting the probability of cell death [10]. This could be part of the mechanism involved in BCG-NO radio-sensitization given that augmented DNA-double strand breaks were observed in the group BCG + IR.

The absence of radio-sensitization of BCG in the 2D model, could be related to the concentration of oxygen. In a 2D model in normoxia the NO generated by BCG is rapidly transformed to soluble nitrite in the medium [21], while in the 3D model nitrite could be accumulated inside the spheroid and be reduced to NO in hypoxic areas by mammal enzymes such as xanthine oxidase [22] and cytochrome C oxidase [23] that acquire this ability in low oxygen



Metastasis	Control	BCG	IR	BCG+IR
Absence	0 (0%)	2 (28%)	1 (12%)	4 (44%)
Presence	5	5	7	5
Total	5	7	8	9

Fig. 5. BCG sensitized MB49-I tumors to IR in vivo. Furthermore, the combined treatment induced the activation of caspase-3 and decreased metastatic spread. C57BL/6 J mice were injected with MB49-I cells subcutaneously into the left flank and tumors were allowed to grow. Animals were then randomly divided into four groups of five mice each: control, BCG, IR or BCG + IR. BCG treatment (0.2 mg in 100 μL of saline solution) was given as an intratumor injection. IR was administered in fractionated doses of 3Gy, 2 days/week reaching a total dose of 18 Gy. After treatment, remnant tumors were resected**. A**, experimental design. **B**, Clonogenic assays were performed to assess radiosensitizing capability of BCG *in vivo*. Cells coming from the disaggregation of *in vivo* treated tumors were counted, plated and cultured for 12 days. Kruskal Wallis Test ($p < 0.01$) and Dunn's post-test, a = $p < 0.01$ difference with control. **C**, an inmunobloting was performed to assess the activation of the apoptosis effector caspase-3, in each of the resected tumors after *in vivo* treatment. Immunobloting for β-actin was used as a loading control. **E**, the presence of lung metastasis at day 44, and **D**, the volume of those metastases were assessed as a measure of systemic disease progression. The combined treatment of BCG and IR showed higher percentage of mice without lung metastasis, and diminished metastatic volume in those mice with metastasis, as compared to control. Kruskal Wallis Test ($p < 0.05$) and Dunn's post-test, a = $p < 0.05$ difference from control.

condition. This explanation, give evidence that higher levels of NO could be available inside the spheroid in the moment of irradiation.

Since *in vitro* BCG radio-sensitizing effect was observed, the next step was to evaluate the effect of these treatments in tumor bearing mice, both at primary tumor level and at a systemic level. To demonstrate the improvement of the combined therapy we performed treatment of established tumors.

At the primary tumor level, the treatment with BCG alone did not show effect over the established tumors, just as it had been reported by other authors [24,25]. This lack of effect was expected because one of the limitations of BCG or any immunotherapy is the tumor burden. Large tumor masses are not inhibited by any immunotherapy. For successful therapy, close contact between the *Mycobacterium* and cancer cells is required.

On the other hand, we previously reported a dose dependent cytotoxic effect of BCG on non invasive MB49 cell line [18], by the contrary, in invasive MB49-I cells line [17,26] no cytotoxic effect of BCG was detected *in vitro*.

With regard to IR, it had local effect, showing tumor growth inhibition up to day 17 when it was given as only treatment. Nevertheless, after then, tumors started growing again and at the end of the treatment no significantly caspase 3 raise was found.

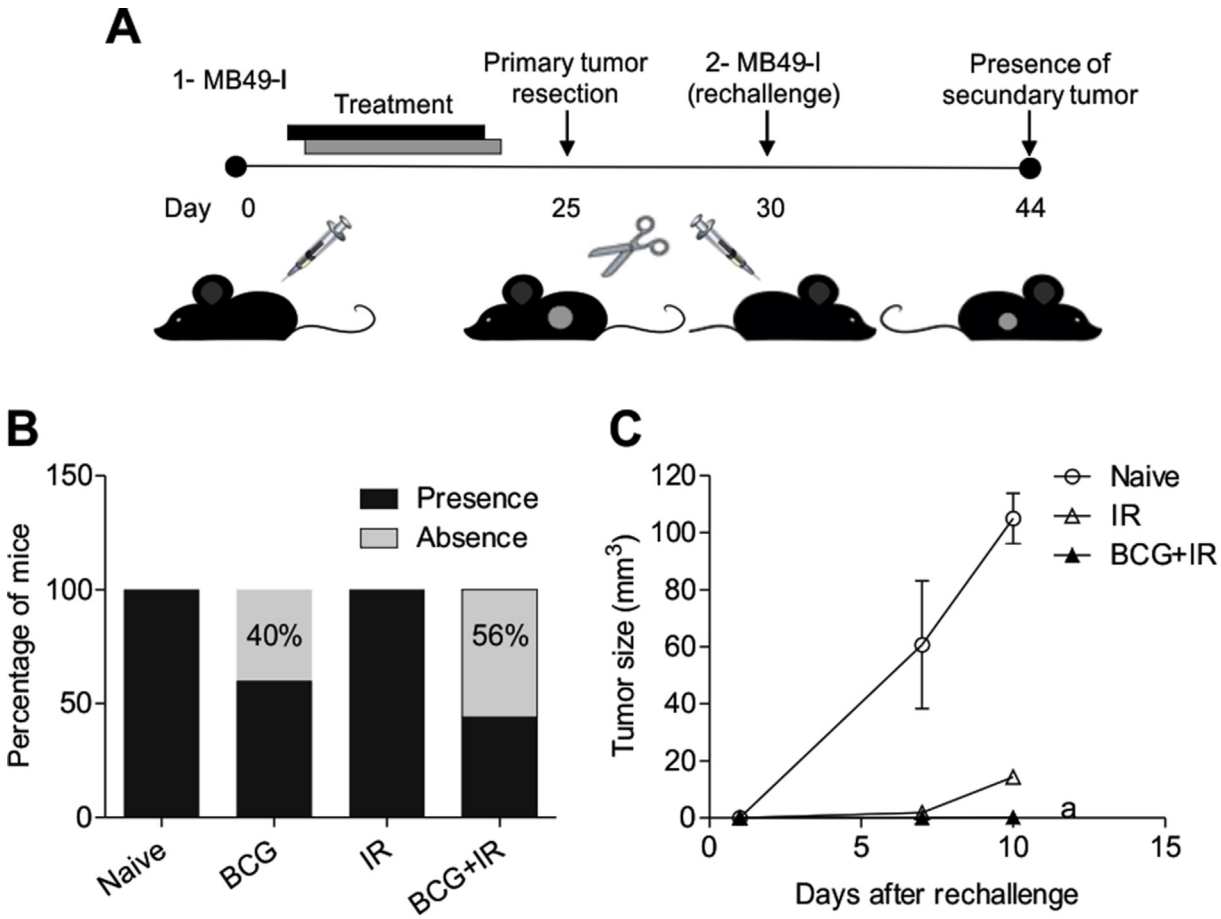


Fig. 6. BCG treatment improved abscopal effect to IR. C57BL/6 J mice were injected with MB49-I cells subcutaneously and treated with BCG, IR or BCG + IR. After treatment, remnant tumors were resected and five days later, mice were rechallenged with a second MB49-I injection in the opposite flank of the first tumor. **A**, experimental design. **B**, The appearance of a second tumor as a consequence of the second MB49-I injection was evaluated as a measure of systemic response, where the rejection of such tumors was assumed as presence of abscopal effect. **C**, effect of first tumor treatment on second tumor growth after rechallenge. Kruskal Wallis Test ($p < 0.01$) and Dunn's post-test, a = $p < 0.01$ difference from naïve group.

Clonogenic assays revealed that remaining tumor cells retained some of their clonogenic survival, suggesting they could be responsible for recurrence and treatment failure. In order to characterize the mechanism of tumor growth inhibition induced by IR, further assays should be performed before the day 17.

On the other hand, tumors from BCG + IR group reached maximum tumor size average at day 13. Thereafter, the growth curve showed negative slope indicating cell death beat repopulation. This effect could be due to the radio-sensitizing effect of BCG observed *in vitro*, and to the activation of an anti-tumor immune response. Clonogenic assays performed with the remaining tumor cells showed that cells from tumors treated with BCG + IR had 100 times lower clonogenic viability than cells from untreated tumors. This suggests that the probability of recurrence after IR could be reduced by this treatment. As a mechanism of action, BCG + IR augmented the activation of caspase-3, suggesting apoptosis as one of the possible death mechanisms involved in tumor shrinkage. It has been reported that in response to low doses of radiation, NO promotes p53 nuclear retention, sensitizing cells to undergo apoptosis [27]. In addition, other authors had recently reported that BCG cell wall cytoskeleton had radio-sensitizing effects on colon cancer cells *in vitro* and *in vivo;* inducing autophagy as cell death [28].

In contrast to the positive effects observed when combining BCG with IR, some authors had reported that BCG treatment inhibited both apoptosis induced by camptothecin and cell death after administration of mitomycin C [29,30] giving evidence about the differences between combining BCG with IR or CT.

Once we proved *in vivo* that BCG was able to improve the local response of invasive BCa to IR, the last step was to evaluate the presence of systemic response by measuring metastases and the rejection of a second tumor.

In BCa, muscle invasion is associated to a high risk of development of distant metastases. In this case, Cisplatin is the first line therapy, but despite the high initial response, almost all patients succumb to distant disease. Immunotherapy has emerged as one of the most promising treatments for metastatic BCa after cisplatin progression [31]. Furthermore, immunotherapy could be used as part of a conservative treatment for invasive BCa, in order to avoid progression to metastases. As it has been reported, IR therapy can lead to the acquisition of a tumor-specific immune phenotype [11], and this effect can be improved by the local administration of exogenous pathogenic signals [11] such as BCG. Given these, here we suggest the combination of BCG and IR as an “anti-tumor vaccine” where IR could act as a tumor antigen generator and BCG as a stimulator of those antigens presentation in an inflammatory context.

According to our present results, when MB49-I primary tumors were resected, all control mice developed large lung metastases. Otherwise, the treatment with IR alone did not seem to be enough

for tumor bearing mice to acquire tumor-specific immune phenotype, given neither lung metastases remission nor abscopal effect after re-challenge were observed. On the other hand, it is noteworthy that almost fifty percent of mice, which received BCG + IR and were subsequently resected, were lung metastasis free. Furthermore, almost sixty percent of these mice rejected the re-challenge, indicating abscopal effect. These results support that the combination of BCG + IR proposed, can lead to the acquisition of tumor-specific immune phenotype, which could not only improve the local control but also the response against metastatic disease.

BCG has been approved since 1990 [13] as an immunological adjuvant in the development of many anti-tumor vaccines. In 2013 a phase I study on combined therapy with proton-beam radiotherapy and *in situ* tumor vaccination with a formulation which included BCG concluded that the treatment was feasible and safe for locally advanced recurrent hepatocellular carcinoma [32,33]. The same conclusion was achieved with a group of patients with advanced breast cancer treated with autologous tumor lysate/BCG immunotherapy as an adjuvant to conventional breast cancer therapy [29].

Finally, in this work we used a murine model with intratumor injections of viable BCG as a simulation of vesical instillation, currently used in patients with noninvasive tumors. However, we are aware that intra tumor injection of viable BCG is not applicable in patients, since can cause BCG dissemination and sepsis. We propose bladder instillation of BCG as an enhancer of IR in conservative invasive bladder cancer therapy. Eventually, other formulations of BCG and other ways of administration could be investigated.

5. Conclusion

BCG treatment concomitant with IR therapy could be useful for the treatment of patients with invasive BCa, through an increase in the probability of local control due to the radio-sensitizing effect of NO induced by BCG, and through an improvement of local and systemic immune anti-tumor response.

Conflict of interest statement

Any authors have conflict of interest with this study and have no personal relationships with other people or organization that could influence their work.

Acknowledgements

This study was supported by funding from Instituto de Oncología “Ángel H. Roffo” - Universidad de Buenos Aires; Escuela Técnica ORT and Centro Médico VIDT SRL.

We are grateful to the Radiotherapy Service of Instituto “Ángel H. Roffo” and Dr. Horacio Blanco and Adriana Paz from Institute Curie from Buenos Aires, Argentina for helping us with the irradiation procedure. We thank Dra. Claudia Arguelles from Instituto Nacional de Producción de Biológicos, for kindly providing BCG. Also, thank Lina Marino and Adriana Sorge from Immunopathology and Bacteriology Departments, respectively, of Instituto Ángel H. Roffo, for performing immunohistochemistry assays and Ziehl-Neelsen staining. We also thank Inés Kletzky for revising our English version.

References

[1] A. Jemal, et al., Global cancer statistics, CA Cancer J. Clin. 61 (2) (2011) 69–90.
[2] D.S. Kaufman, W.U. Shipley, A.S. Feldman, Bladder cancer, Lancet 374 (9685) (2009) 239–249.
[3] J.P. Stein, et al., Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients, J. Clin. Oncol. 19 (3) (2001) 666–675.
[4] O. Caffo, et al., Trimodality treatment in the conservative management of infiltrating bladder cancer: a critical review of the literature, Crit. Rev. Oncol. Hematol. 86 (2) (2013) 176–190.
[5] H. Harada, How can we overcome tumor hypoxia in radiation therapy? J. Radiat. Res. 52 (5) (2011) 545–556.
[6] P.J. Hoskin, et al., Radiotherapy with concurrent carbogen and nicotinamide in bladder carcinoma, J. Clin. Oncol. 28 (33) (2010) 4912–4918.
[7] E.O. Sandes, et al., Expression of inducible nitric oxide synthase in tumoral and non-tumoral epithelia from bladder cancer patients, Nitric Oxide 12 (1) (2005) 39–45.
[8] G. Shah, et al., iNOS expression and NO production contribute to the direct effects of BCG on urothelial carcinoma cell biology, Urol. Oncol. 32 (1) (2014), p. 45 e1-9.
[9] M. De Ridder, et al., Hypoxic tumor cell radiosensitization: role of the iNOS/NO pathway, Bull. Cancer 95 (3) (2008) 282–291.
[10] J. Scicinski, et al., NO to cancer: The complex and multifaceted role of nitric oxide and the epigenetic nitric oxide donor, RRx-001, Redox Biol. 6 (2015) 1–8.
[11] C.M. Ludgate, Optimizing cancer treatments to induce an acute immune response: radiation Abscopal effects, PAMPs, and DAMPs, Clin. Cancer Res. 18 (17) (2012) 4522–4525.
[12] E.F. Stamell, et al., The abscopal effect associated with a systemic anti-melanoma immune response, Int. J. Radiat. Oncol. Biol. Phys. 85 (2) (2013) 293–295.
[13] S.G. Patel, et al., Intravesical therapy for bladder cancer, Expert Opin. Pharmacother. 16 (6) (2015) 889–901.
[14] A. Bohle, S. Brandau, Immune mechanisms in bacillus Calmette-Guerin immunotherapy for superficial bladder cancer, J. Urol. 170 (3) (2003) 964–969.
[15] E. Morcos, et al., Bacillus Calmette-Guerin induces long-term local formation of nitric oxide in the bladder via the induction of nitric oxide synthase activity in urothelial cells, J. Urol. 165 (2) (2001) 678–682.
[16] E.O. Sandes, et al., Inducible nitric oxide synthase and PPARgamma are involved in bladder cancer progression, J. Urol. 188 (3) (2012) 967–973.
[17] C. Lodillinsky, et al., Novel invasive orthotopic bladder cancer model with high cathepsin B activity resembling human bladder cancer, J. Urol. 182 (2) (2009) 749–755.
[18] V. Alvarez, et al., Inhibition of bacillus Calmette-Guerin-induced nitric oxide in bladder tumor cells may improve BCG treatment, Int. J. Mol. Med. 16 (4) (2005) 565–571.
[19] C. Lodillinsky, et al., Bacillus Calmette Guerin induces fibroblast activation both directly and through macrophages in a mouse bladder cancer model, PLoS One 5 (10) (2010) e13571.
[20] Y. Langle, et al., Role of peroxisome proliferator activated receptor-gamma in bacillus Calmette-Guerin bladder cancer therapy, J. Urol. 188 (6) (2012) 2384–2390.
[21] L. Policastro, et al., Selective radiosensitization by nitric oxide in tumor cell lines, Cancer Lett. 248 (1) (2007) 123–130.
[22] T.M. Millar, et al., Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions, FEBS Lett. 427 (2) (1998) 225–228.
[23] P.R. Castello, et al., Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes, Cell Metab. 3 (4) (2006) 277–287.
[24] D. Chassoux, J.C. Salomon, Therapeutic effect of intratumoral injection of bcg and other substances in rats and mice, Int. J. Cancer 16 (4) (1975) 515–525.
[25] J. Tai, et al., Inhibition of pulmonary metastases of B16 melanoma with irradiated tumor cells and BCG, Cancer Immunol. Immunother. 15 (1) (1983) 47–53.
[26] D. Belgorosky, et al., Inhibition of nitric oxide is a good therapeutic target for bladder tumors that express iNOS, Nitric Oxide 36C (2013) 11–18.
[27] X. Wang, A. Zalcenstein, M. Oren, Nitric oxide promotes p53 nuclear retention and sensitizes neuroblastoma cells to apoptosis by ionizing radiation, Cell Death Differ. 10 (4) (2003) 468–476.
[28] J.M. Yuk, et al., Bacillus calmette-guerin cell wall cytoskeleton enhances colon cancer radiosensitivity through autophagy, Autophagy 6 (1) (2010) 46–60.
[29] S. Holla, et al., Mycobacterium bovis BCG promotes tumor cell survival from tumor necrosis factor-alpha-induced apoptosis, Mol. Cancer 13 (2014) 210.
[30] F. Chen, et al., Bacillus Calmette-Guerin inhibits apoptosis in human urothelial carcinoma cell lines in response to cytotoxic injury, J. Urol. 178 (5) (2007) 2166–2170.
[31] T.L. Rose, M.I. Milowsky, Improving systemic chemotherapy for bladder cancer, Curr. Oncol. Rep. 18 (5) (2016) 27.
[32] M. Abei, et al., A phase I study on combined therapy with proton-beam radiotherapy and in situ tumor vaccination for locally advanced recurrent hepatocellular carcinoma, Radiat. Oncol. 8 (2013) 239.
[33] J. Convit, et al., Autologous tumor lysate/Bacillus Calmette-Guerin immunotherapy as an adjuvant to conventional breast cancer therapy, Clin. Transl. Oncol. 17 (11) (2015) 884–887.