

Radiotherapy-Activated Hafnium Oxide Nanoparticles Produce Abscopal Effect in a Mouse Colorectal Cancer Model

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Purpose: Despite tremendous results achieved by immune checkpoint inhibitors, most patients are not responders, mainly because of the lack of a pre-existing anti-tumor immune response. Thus, solutions to efficiently prime this immune response are currently under intensive investigations. Radiotherapy elicits cancer cell death, generating an antitumor-specific T cell response, turning tumors in personalized in situ vaccines, with potentially systemic effects (abscopal effect). Nonetheless, clinical evidence of sustained anti-tumor immunity as abscopal effect are rare.

Methods: Hafnium oxide nanoparticles (NBTXR3) have been designed to increase energy dose deposit within cancer cells. We examined the effect of radiotherapy-activated NBTXR3 on anti-tumor immune response activation and abscopal effect production using a mouse colorectal cancer model.

Results: We demonstrate that radiotherapy-activated NBTXR3 kill more cancer cells than radiotherapy alone, significantly increase immune cell infiltrates both in treated and in untreated distant tumors, generating an abscopal effect dependent on CD8⁺ lymphocyte T cells.

Conclusion: These data show that radiotherapy-activated NBTXR3 could increase local and distant tumor control through immune system priming. Our results may have important implications for immunotherapeutic agent combination with radiotherapy.

Keywords: CD8⁺ T cells, antitumor immune response, TILs, NBTXR3, radioenhancer

Introduction

Since decades, radiotherapy (RT) is one of the main treatments for many cancer types, employed to locally destroy cancerous cells and achieve substantial tumor debulking. About 60% of all cancer patients will receive irradiations as part of their treatment.¹ Beyond the ability of RT to kill cancer cells by production of free radicals and generation of single and double-strand breaks in DNA,² preclinical and clinical studies have demonstrated that RT can prime an immunomodulatory response.^{3,4} For example, RT can stimulate MHC class I expression on cancer cells,⁵ induce the immunogenic cell death^{6,7} important for CD8⁺ cytotoxic T lymphocyte activity,^{8,9} and also activate expression of various pro- and anti-inflammatory cytokines and adhesion molecules, allowing recruitment and activation of both innate and adaptive immune cells into the tumor.¹⁰ Unfortunately, RT rarely produces a sustained anti-tumor response as immune escape frequently occurs with tumor recurrence.^{11,12} Moreover, the so-called “abscopal effect” which corresponds to reduction of metastatic burden outside the irradiated area

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is rarely observed after radiotherapy.^{13–16} Finally, because of toxicity to surrounding healthy tissue, the maximum dose of irradiation will always be limited.

The high electron density of functionalized hafnium oxide nanoparticles (NBTXR3)^{17,18} allows a high probability of interaction with incoming ionizing radiation, increasing energy dose deposit within cells. Thanks to this primary physical mode of action, we have previously reported in preclinical studies the increased ability of RT-activated NBTXR3 (NBTXR3+RT) to induce cancer cell death and control tumor growth, compared to RT alone.^{17,19} We recently reported that NBTXR3+RT can enhance cGAS-STING pathway activation in human colorectal cancer cells.²⁰ Further, NBTXR3+RT demonstrated clinically meaningful benefit for patients with locally advanced Soft Tissue Sarcoma compared to RT alone, in the randomized controlled Phase II/III Act.in.Sarc study (NCT02379845).²¹

In this article, we evaluated the impact of NBTXR3+RT on the anti-tumor immune response. We demonstrated the ability of NBTXR3 to kill more cancer cells and to induce an abscopal effect. Examination of immune cell infiltrates in treated and untreated tumors revealed that NBTXR3+RT can increase the infiltrates of CD8⁺ T cells in both tumors. We also show that the abscopal effect produced by NBTXR3+RT is dependent on CD8⁺ T-cells. Taken together, these results indicate that NBTXR3+RT can efficiently prime an anti-tumor immune response, which could have important implications for the use of NBTXR3+RT with immunotherapy.

Materials and Methods

Cells and Reagents

CT26.WT cells were purchased from the ATCC (#CRL-2638), cultivated according to the provider's recommendations and screened for mycoplasma (CleanCells). NBTXR3 (Nanobiotix) is a sterile suspension of functionalized HfO₂ spherical nanoparticles with a size centered on 50 nm, bearing a marked negative surface charge (–50 mV) in aqueous solution at pH 6–8.¹⁷

Mice

Six-week-old BALB/c female mice (Janvier Labs) were maintained under pathogen-free conditions in the animal facility at Gustave Roussy Institute (Villejuif, France). All animal experiments were carried out in compliance with French and European laws and regulations (European Directive 2010/63 EU). The local institutional animal

ethics board and French Ministère de la Recherche approved all mouse experiments (permission numbers: 2016_031_4340 and 2016_129_8344).

Irradiation

For in vitro experiments, X-ray irradiations were delivered using an X-Ray generator at 125 kV (CellRad, Faxitron). For in vivo assays, tumor irradiations were performed with a 200 kV Varian NDI 226 X-ray irradiator. Selective irradiation of the tumor on mice was performed by the interposition of a lead shield, allowing full protection of the rest of the body, including proximal lymph nodes.

Cell Death Analysis

CT26.WT cells were seeded in duplicate in 6-well plates incubated overnight with or without 400 μ M NBTXR3 nanoparticle suspension, then irradiated with a single fraction of 2, 3, 4, 5 or 6 Gy. After 48 h, cells were harvested, centrifuged then resuspended in 100 μ L of 1 \times Binding Buffer per 10⁶ cells, containing 10 μ L Annexin V Conjugated to FITC (Annexin V-FITC Kit, Miltenyi Biotec, Germany). Cells were incubated in the dark at room temperature for 15 min; then, 1 μ g/mL propidium iodide was added to the cell suspension, followed by flow cytometry analysis on Accuri C6 (BD Biosciences).

Intratumoral Bioavailability and Persistence of NBTXR3

Mice were subcutaneously injected into the flank with 3.10⁵ CT26.WT cells to generate a single tumor. Once tumor volume was comprised between 50 and 120 mm³, NBTXR3 suspension equivalent to 25% of the baseline tumor volume was delivered by intratumoral injection. The next day, intratumoral NBTXR3 bioavailability was confirmed by μ CT-scan (Voxcan, France). One-week post-i.t., a second CT-scan was performed to confirm NBTXR3 intratumoral persistence.

In vivo Experiments

For the abscopal assay, 3.10⁵ CT26.WT cells were subcutaneously injected into both flanks of mice on the same day. Once the tumor had grown (50 to 120 mm³), mice were randomized to the different groups. A volume of NBTXR3 suspension (or vehicle) corresponding to 25% of the baseline tumor volume was delivered intratumorally into the right flank tumor only (ie, the left flank tumor was untreated). After 24 h, the right tumors were irradiated

with 4Gy per fraction for 3 consecutive days. Length (L) and width (W) of tumors were measured 2–3 times per week using a digital caliper. Tumor volumes were calculated using the formula ($L \times W^2/2$). To evaluate the role of CD8⁺ T cell, an abscopal assay was implemented as previously described. The CD8⁺ T cells were depleted in some mice treated with NBTXR3+RT, by intraperitoneal injections of anti-CD8 (Ref. BP0004-1, clone 53-6.7, BioXcell) (100 µg/mice) at D-1, D1, D4, D7, D11 and D14. For both experiments, mice were sacrificed when one tumor reached 800mm³.

Immunohistochemistry and Digital Pathology Analysis

For immunohistochemistry analyses, the same schedule than for abscopal assays was followed, except that animals were sacrificed 72 h after the last dose of irradiation. Treated and untreated tumors were immediately excised then fixed. For each tumor, 3 slices of 4 µm from FFPE blocks (first third, middle and last third of the tumor) were put on the same slide and slices were stained using specific antibodies raised against CD4 (#50134-R0001, Interchim), CD8 (#AB203035, Abcam) or CD68 (#AB125212, Abcam) on Ventana Discovery XT autostainer and stained by haematoxylin/eosin/safran on Leica ST5020 multistainer. For digital pathology analysis, each stained slide was scanned with Aperio AT Turbo x40 (Excilone, France).

Statistical Analysis

In vitro studies have been independently repeated at least three times. Results are expressed as mean ± SEM. Normality distribution of values was assessed by Shapiro–Wilk normality test. Experiments with normal distribution were analyzed by two-tailed *t*-test. Experiments with non-normal distribution were analyzed by Mann–Whitney test. A *p* value <0.05 was considered statistically significant. For in vivo studies, mean tumor volume was calculated for each group and used for drawing growth curves. The overall survival has been determined by the analysis of Kaplan–Meier curves and calculation of the median survival. Statistical analyses of mean tumor growth curves and Kaplan–Meier curves were performed using Two-Way ANOVA test and Log-rank (Mantel–Cox) test, respectively. Data are reported as means ± SEM. The software GraphPad Prism 7[®] v.7.04 was used for graph plotting and biostatistics.

Results

NBTXR3+RT Efficiently Kill Cancer Cells

We first examined the ability of NBTXR3+RT to kill cancer cells, compared to RT alone. The different cellular physiological states (eg, viability, early apoptosis, early necrosis and late apoptosis/necrosis) were measured 48h after treatment by Annexin V-FITC/propidium iodide staining and flow cytometry (Figure 1). For cells treated with NBTXR3 alone, viability (98.1%±0.65) was the same than for untreated control cells (98.1%±0.37), showing the non-toxicity of the nanoparticles. Compared to RT alone, treatment with NBTXR3+RT led to a significant decrease in cell viability due to enhancement of early apoptosis, early necrosis and late apoptosis/necrosis (Figure 1A–D). The effect of NBTXR3+RT was particularly marked for early apoptosis from 2Gy (Figure 1C). Interestingly, the decrease of cell viability achieved with NBTXR3+4Gy was greater than 6Gy alone, highlighting the radioenhancement ability of NBTXR3. These results are in good accordance with our previously published results of clonogenic assay with various human cancer cells.¹⁹

NBTXR3 Remains in the Tumor

In patients, NBTXR3 is delivered via intratumoral injection. To evaluate the dispersion and persistence of these nanoparticles in our in vivo tumor model, mice bearing a CT26.WT subcutaneous tumor received an intratumoral injection of NBTXR3. Due to the high density of hafnium that composes NBTXR3, the nanoparticles are easily detectable by Micro Computed Tomography (µ-CT) scan, within tissues (Figure 2A). The day after injection, µ-CT scan showed that nanoparticles were distributed in a large part of the tumor. One week after the injection, these nanoparticles remained in the tumor of the same animal, demonstrating the good the persistence of the nanoparticles in the tumor.

NBTXR3+RT Produces an Abscopal Effect

To evaluate the ability of NBTXR3+RT to produce an abscopal response, we used mice bearing subcutaneous tumor on both flanks. The right tumors were treated with or without NBTXR3 then irradiated (or not), whereas the left tumor remained untreated. The treated tumor growth curves for RT and NBTXR3+RT groups were similar, and clearly showed the effect of the RT (Figure 2B, left panel). Despite the clear impact of RT alone on treated tumor growth curve, no abscopal effect has been produced, as the untreated tumor growth curve

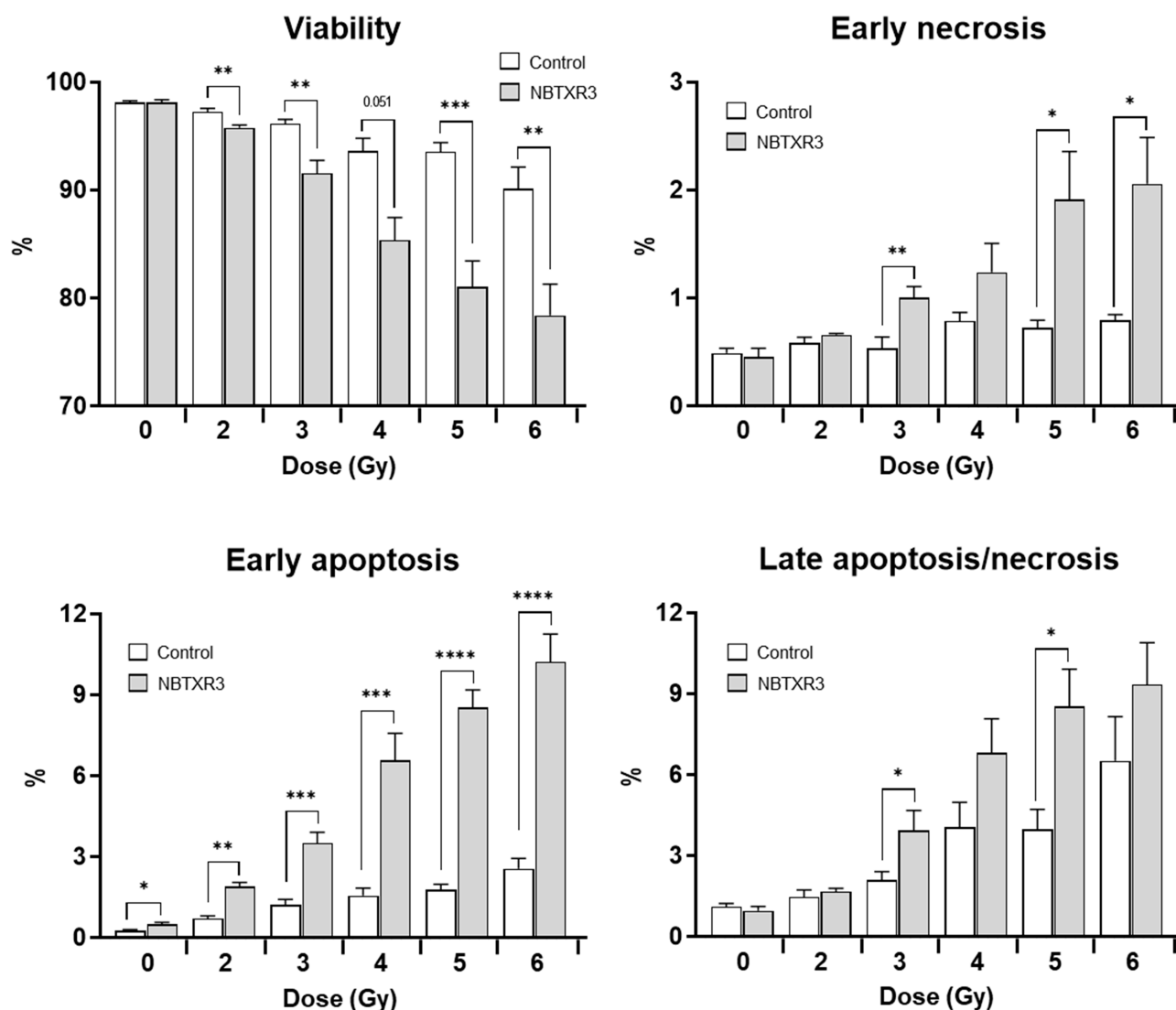


Figure 1 NBTXR3 activated by RT results kill more cancer cells than RT alone. Percentages of (A) viability, (B) early necrosis, (C) early apoptosis and (D) late apoptosis/necrosis were assessed 48h after RT by Annexin V-FITC/PI staining in CT26.WT cells treated or not with 400 μ M of NBTXR3 and irradiated with increasing doses of RT. Presented data were obtained from three independent experiments (n=3) performed in duplicate. Data are represented as mean \pm SEM. Statistical test: two-tailed t-test (C, D) or Mann-Whitney test (A, B). * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001.

for this group was identical to controls (Figure 2B, right panel). In stark contrast, a significant abscopal effect was obtained in mice treated with NBTXR3+RT (p <0.001). In addition, a significant lengthening of the lifespan of animals treated with NBTXR3+RT was obtained (median survival 19.5 vs 14 for NBTXR3+RT and RT alone, respectively, p <0.01), while survival for the other groups was similar (Figure 2C and D).

NBTXR3+RT Increases Immune Cell Infiltrates in Both Tumors

To further investigate the mechanism driving the NBTXR3+RT abscopal effect, we repeated the experiment by sacrificing the animals 3 days after the last fraction of irradiation and

measured CD4+, CD8+ and CD68+ cell densities in both tumors (Figure 3A). For CD4+, only irradiated tumors showed a marked difference compared to control groups. Nonetheless, no significant difference between RT and NBTXR3+RT has been observed. In contrast, significant increases in CD8+ and macrophages (CD68+) infiltrates (p <0.05) were observed in both treated and distant untreated tumors in NBTXR3+RT mice, compared to RT alone.

NBTXR3+RT Abscopal Effect Is Dependent on CD8+ Cells

To determine the role of CD8+ cells in the abscopal effect produced by NBTXR3+RT, we repeated an abscopal assay, including a group of mice previously depleted of their

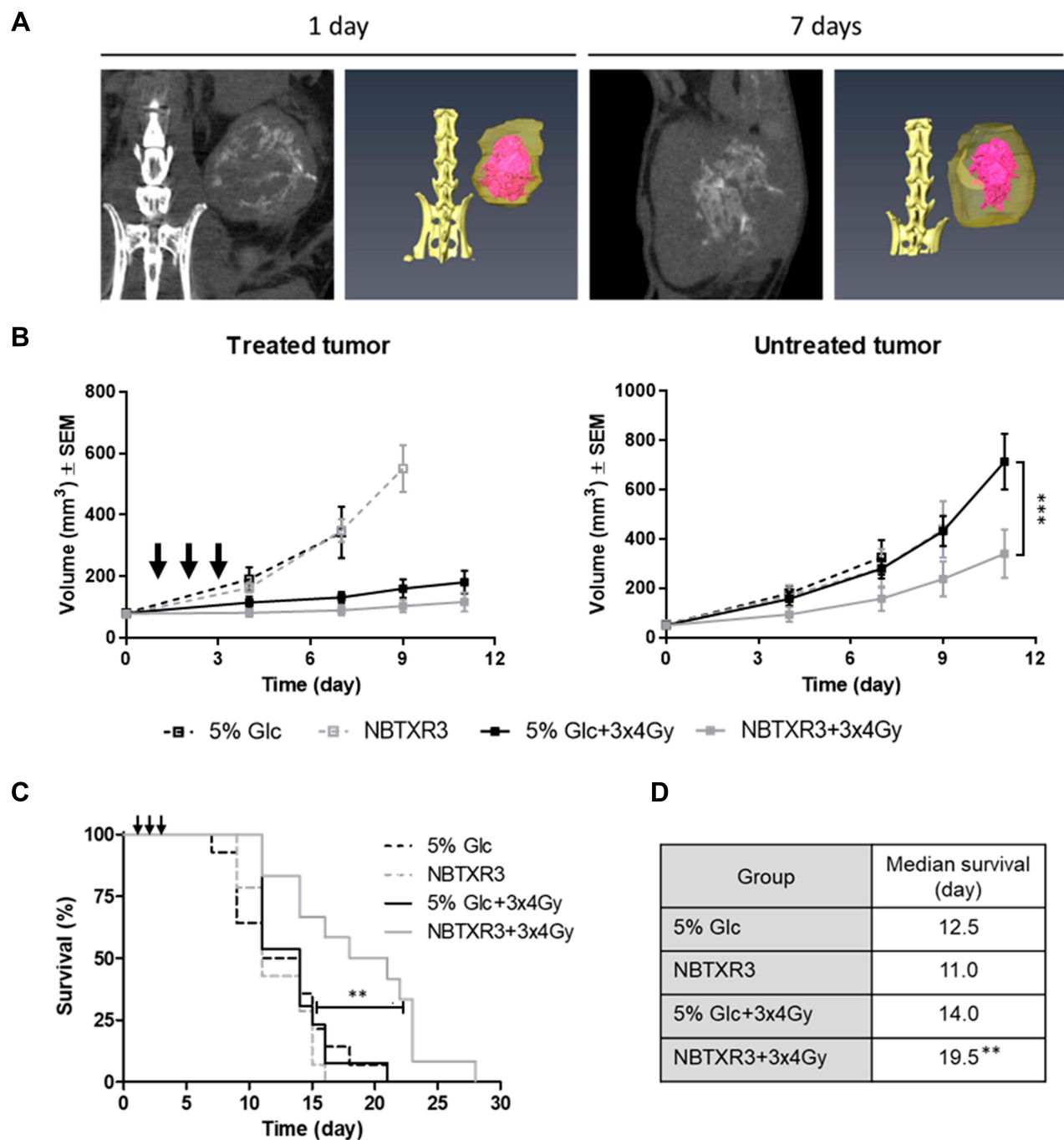


Figure 2 Distribution and persistence of NBTXR3 after intratumoral injection was imaging by μ CT performed 1 day (left panel) and 7 days (right panel) after NBTXR3 injection. Pink, NBTXR3; brown, tumor (A). For abscopal experiments (B) tumor growth curves of treated (left panel) and distant untreated (right panel) tumors (C) survival curves and (D) median survival were obtained from two independent experiments (n=2), with 12–15 mice per group. Arrows indicate radiotherapy sessions (4 Gy). Statistical analysis: two-way ANOVA test (B) or Log-rank (Mantel-Cox) test (C). ** $p < 0.01$; *** $p < 0.001$.

CD8⁺ cells before treatment with NBTXR3+RT. For the groups not depleted in CD8⁺ cells, the results were similar to those previously obtained, with a marked abscopal effect for the group of mice treated with NBTXR3+RT (Figure 3B). Interestingly, depletion of CD8⁺ cells completely abolished the abscopal effect. This shows that CD8

+ cells drive the abscopal effect induced by RT-activated NBTXR3.

Discussion

In this article, we explored the impact of NBTXR3 activated by RT on the anti-tumor immune response. We

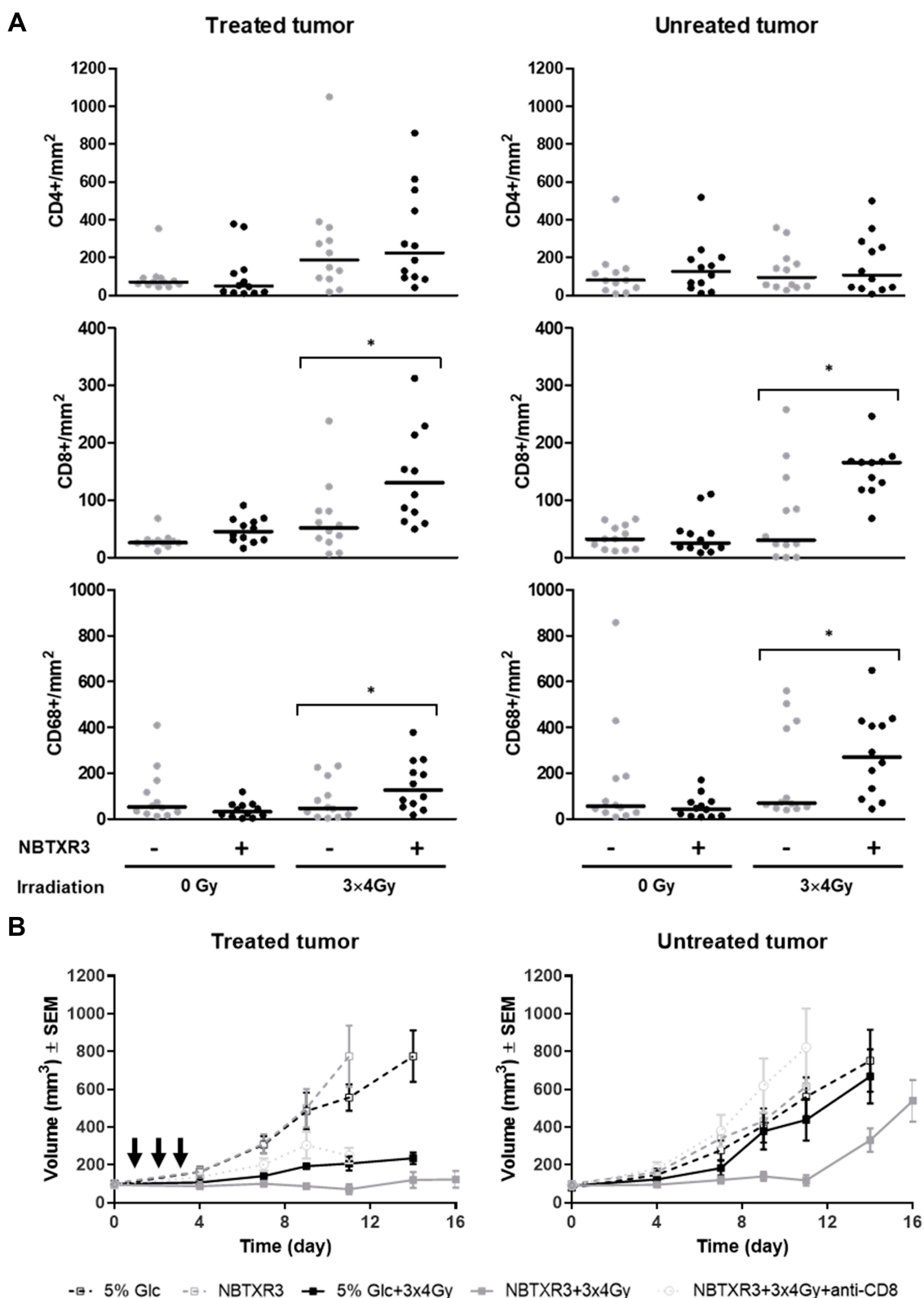


Figure 3 Modulation of immune cell infiltrates by NBTXR3 activated by RT. Density of CD4+ (upper panel), CD8+ (middle panel) and CD68+ cells (lower panel) infiltrates in treated tumors (left panel) and in distant untreated tumors (right panel) were analyzed by IHC. For each tumor, 3 slices from FFPE blocks (first third, middle and last third of the tumor) were put on the same slide and slices were stained using specific antibodies. Presented data were obtained from four mice per group (each dot represents one value). Bars represent median. Statistical test: two-tailed t-test. * $p < 0.05$ (A). Induction of an abscopal effect by NBTXR3+RT is dependent on CD8+ T cells. Tumor growth curves of treated (left panel) and distant untreated (right panel) tumors (8 mice per group). Arrows indicate radiotherapy sessions (4Gy) (B).

demonstrated that NBTXR3 activated by RT more efficiently induced cancer cell death than RT alone, mainly by a significant increase of apoptosis. We also showed that NBTXR3+RT significantly increased necrosis, while RT alone had almost no impact. Interestingly, these results suggest that NBTXR3+RT amplified the main cell death mechanism triggered by RT but was also able to modulate another cell death pathway in these cells. This ability of NBTXR3 to modulate biological pathways set in motion by RT is consistent with our previous work on the enhancement of the cGAS-STING pathway in colorectal cancer cells.²⁰ On the other hand, it is interesting to note that cell death levels induced by the highest doses of radiotherapy alone can be achieved (or even exceeded) with much lower radiotherapy doses, in the presence of NBTXR3 (eg, NBTXR3+4Gy vs 6Gy). These data demonstrate NBTXR3 radioenhancement ability, which opens interesting possibilities for RT dose reduction in clinical trials. Indeed, it has been shown by Bonvalot et al²¹ that NBTXR3 had a good safety profile. However, the expected adverse events of RT persist. If a reduced dose of RT with NBTXR3 can be as efficient as a standard dose of RT without NBTXR3, this made it possible to reduce the adverse events due to RT, thus significantly improving the quality of life of the patients.

NBTXR3 is administered by intratumoral injection and it was important to evaluate the distribution and persistence of the nanoparticles in tumors after administration. The μ -CT imaging showed both good distribution and persistence of nanoparticles in the tumor after at least 1 week. These results are in good agreement with previously published data,¹⁷ indicating that nanoparticles will be present at least throughout the duration of RT treatment of *in vivo* studies.

One of the desired effects after RT is the generation of a strong anti-tumor immune response able to generate an abscopal effect. Unfortunately, this effect is rarely observed in patients.²² Based on previous preclinical and clinical results,^{17,19-21} we hypothesized that the radioenhancement effect of NBTXR3 may be strong enough to generate this effect. To test this hypothesis, we used immuno-competent mice carrying a subcutaneous tumor on both flanks. Only right tumors received treatment, while left tumors remained untreated. Thanks to this approach, we demonstrated that tumors treated with NBTXR3+RT produced a significant abscopal response. The generation of this abscopal effect is particularly noteworthy since treated tumor growth control was similar for

RT alone and NBTXR3+RT groups. This suggests that tumor microenvironment modifications triggered by NBTXR3+RT treatment were stronger (and/or different) to prime an anti-tumor immune response able to produce an abscopal effect, compared to radiotherapy alone. This hypothesis is supported by the comparison of immune cell densities in the treated and distant untreated tumors for the different groups. Remarkably, we measured a significant increase in CD8+ infiltrates (known to play a central role in cancer cell destruction) both in treated and untreated tumors of NBTXR3+RT group, compared to RT alone. We also demonstrated that this abscopal effect was highly dependent on CD8+ cells, as their depletion results in the complete abolishment of the abscopal effect.

Conclusion

Taken together, our results clearly demonstrate the benefit of NBTXR3 on the antitumor immune response activation, particularly at the level of adaptive response mediated by CD8+ cells. NBTXR3 activated by RT efficiently transformed the tumor in an *in situ* vaccine, with systemic effect on a distant untreated lesion. As such, NBTXR3 could be an innovative solution to overcome the current limitations of RT. This study may have important implications for the combination of immunotherapeutic agents with radiotherapy.

Abbreviations

RT, radiotherapy; μ CT, micro X-ray computed tomography.

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Disclosure

P. Zhang, A. Darmon, J. Marill, N. Mohamed Anesary, and S. Paris are employees of Nanobiotix. The authors report no other conflicts of interest in this work.

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